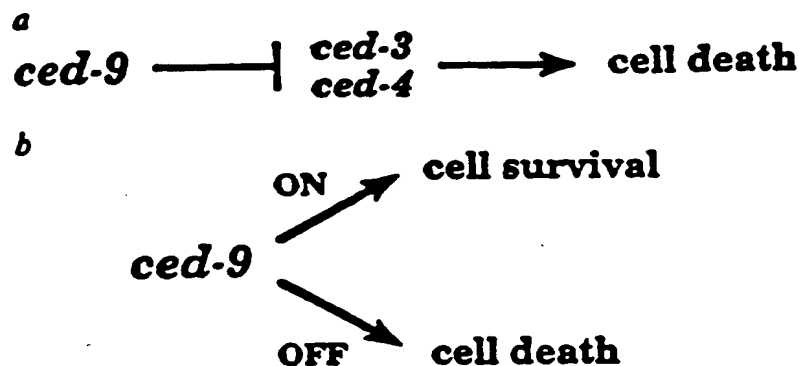




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>C12N 15/12, 15/11, C12Q 1/68</b> <b>C07K 13/00, A61K 31/70, 37/02</b> <b>G01N 33/577</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/25683</b> <b>(43) International Publication Date:</b> 23 December 1993 (23.12.93)
<b>(21) International Application Number:</b> PCT/US93/05651 <b>(22) International Filing Date:</b> 14 June 1993 (14.06.93)  <b>(30) Priority data:</b> 07/898,933                      12 June 1992 (12.06.92)      US 07/927,681                      10 August 1992 (10.08.92)     US  <b>(71) Applicant:</b> MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachussets Avenue, Cambridge, MA 02139 (US).  <b>(72) Inventors:</b> HORVITZ, H., Robert ; 80 Wendell Street, No. 2, Cambridge, MA 02138 (US). HENGARTNER, Michael ; 14 South Russel, No. 1, Boston, MA 02114 (US).	<b>(74) Agents:</b> GRANAHAH, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).  <b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

**(54) Title:** A GENE WHICH PREVENTS PROGRAMMED CELL DEATH

**(57) Abstract**

The present invention relates to genes, referred to herein as cell death-protective genes, which protect cells against programmed cell death by antagonizing the activities of genes which cause cell death. As described herein, a cell death-protective gene from the nematode *Caenorhabditis elegans*, called *ced-9*, has been identified, sequenced, and characterized. *ced-9* is essential for *C. elegans* development and apparently functions by protecting cells which normally live during development from programmed cell death. Mutations which constitutively activate and inactivate the *ced-9* gene are also described. *ced-9* was shown to function by antagonizing the activities of the cell death genes, *ced-3* and *ced-4*. As further described, the protein product of the human oncogene *bcl-2* was found to have a similar sequence to the *ced-9* protein. Methods and agents for both increasing and decreasing the occurrence of cell death are described that are potentially useful for diagnosis, prevention and therapy of diseases and conditions involving cell death; for the treatment of viral, parasitic, and other types of infection; and for killing organisms that are detrimental or potentially detrimental to the environment or to humans, pets, livestock, or agriculture.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

- 1 -

A GENE WHICH PREVENTS PROGRAMMED CELL DEATHBackground

Cell death is a fundamental aspect of animal development. A considerable proportion of the cells that are generated die during the normal development of both vertebrates (Glucksmann, Biol. Rev. Cambridge Philos. Soc. 26:59-86 (1951)) and invertebrates (Truman and Schwartz, Ann. Rev. Neurosci. 7:171-188 (1984)). Cell death plays a role in morphogenesis (e.g., of the eye, secondary palate, heart, nervous system and limbs in vertebrate embryos), metamorphosis (e.g., in moths and other insects), and tissue homeostasis (e.g., of epithelial linings and the thymus), as well as in neuron selection during the establishment of synaptic connections and in sexual dimorphism (reviewed by Ellis et al., Ann. Rev. Cell Biol. 7:663-698 (1991)). Cell death which occurs as a part of normal development will be referred to herein as physiological cell death.

Besides physiological cell death, cell death may occur as a pathological manifestation of disease, in which case it will be referred to herein as pathological cell death (see review by Trump and Mergner (1974), in: The Inflammatory Process, vol. 1, 2nd ed. (eds. Zweifach et al.), Academic Press, New York, pp. 115-257). Cell death can result from a variety of injuries to the cell, including toxins, ischemia (lack of blood supply), hypoxia (lack of oxygen) and infectious agents, as well as from genetic mutations. The major clinical aspects of most degenerative diseases are a consequence of cell death. For example, Huntingtons's disease, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis are marked by degeneration of neurons, while Duchenne muscular dystrophy is characterized by muscle degeneration. In addition, some cancers are thought to be caused by a defect in cell death processes. Thus,

understanding and preventing cell death can be viewed as one of the major goals of biomedical research.

The simple and invariant anatomy and development of the nematode Caenorhabditis elegans have made it an attractive system for the study of cell death. Because C. elegans is small, cellularly simple and transparent, Nomarski differential interference microscopy can be used to observe individual cells throughout development. As a result, the complete cell lineage of C. elegans, from zygote to adult, has been elucidated (Sulston and Horvitz, Dev. Biol. 82:110-156 (1977); Kimble and Hirsh, Dev. Biol. 70:396-417 (1979); Sulston et al., Dev. Biol. 100:64-119 (1983)).

Cell death is an important component of the development of C. elegans: during the development of the adult hermaphrodite, the generation of 816 nongonadal cells is accompanied by the generation and subsequent deaths of an additional 131 cells. Cell death appears to be an integral part of the differentiation of a variety of tissues. The pattern of cell deaths is essentially invariant among different animals, i.e., the same set of cells die at the same developmental time. In addition, a vast majority of cell deaths in C. elegans does not appear to be initiated by interaction with surrounding cells or diffusible factors.

Genetic analysis has identified many genes that affect programmed cell death in C. elegans (reviewed by Ellis et al. (1991) supra). The activities of two genes, ced-3 and ced-4, seem to be required for the onset of almost all C. elegans programmed cell deaths (Ellis and Horvitz, Cell 44:817-829 (1986)). Mutations in ced-3 and ced-4 block essentially all programmed cell deaths. In ced-3 and ced-4 mutants, cells that normally undergo programmed cell death instead survive, differentiate and even function (Ellis and Horvitz

-3-

(1986) supra; Avery and Horvitz, Cell 51:1071-1078 (1987); White et al., Phil. Trans. R. Soc. Lond. B. 331:263-172 (1991)). Genetic analyses indicate that ced-3 and ced-4 genes most likely act within dying  
5 cells; this suggests that of these genes are expressed within dying cells and either encode cytotoxic molecules or control the activities of cytotoxic molecules (Yuan and Horvitz, Dev. Biol. 138:33-41 (1990)).

Relatively little is known about the mechanism of  
10 cell death. Initiation of cell death occurs in response to a variety of signals. External injuries and cytotoxic agents cause cells to die. Endocrine signals trigger cell death during insect metamorphosis, thymocyte death and regression of the prostate in the  
15 male rat after castration. Lack of neuronal growth factors is suspected to be the cause of certain neuronal cell deaths during vertebrate development and may also be the cause of cell deaths in certain neurodegenerative diseases. A specific protein, Mullerian inhibiting  
20 substance, is responsible for the regression of the Mullerian duct during the development of male mammals. In addition, genetically programmed cell deaths which occur apparently autonomously of cell-cell interaction or diffusible factors are observed in C. elegans and  
25 other invertebrates. (Truman and Schwartz, Neuro. Comm. 1:66-72 (1982); Cohen and Duke, J. Immunol. 132:38-42 (1984); Isaacs, Prostate 5:545-557 (1984); Martin et al., J. Cell. Biol. 106:829-844 (1988); Oppenheim and Prevet, Neurosci. Abstr. 14:368 (1988); Beal et al.,  
30 Nature 321:168-171 (1986); Birkmayor and Hornykiewicz, Advances in Parkinsonism, Fifth International Symposium on Parkinson's Disease, Vienna, Roche, Basle, 1976; Lagsto et al., Science 219:979-980 (1983); Rossor, Lancet 2:1200-1204 (1982); Biel et al., Science 229:289-  
35 291 (1985); Cosi et al., in: Advances in Experimental

-4-

Medicine and Biology, vol. 209, Plenum Press, New York, 1987; Bonilla et al., Cell 54:447-452 (1988); Picard and Josso, Biomedicine 25:147-150 (1976)).

Cell deaths also vary morphologically. Two major categories of cell deaths have been established based on morphological features (Kerr et al., Br. J. Cancer 26:239-257 (1972)). The first type of cell death, called necrosis, is characterized by cellular swelling, rupture of plasma and internal membranes, and eventual leakage of cellular contents into the extracellular space. The second, called apoptosis, involves progressive condensation of cytoplasm and nuclear chromatin and eventual fragmentation of cellular membranes into 'apoptotic bodies', which are usually digested by macrophages or adjacent epithelial cells. Necrosis is most often a manifestation of certain pathological conditions, e.g., injury by complement (Hawkins et al., Am. J. Pathol. 68:255-288 (1972)), hypoxia (Jennings et al., Am. J. Pathol. 81:179-198 (1975)), or exposure to a variety of toxins (McLean et al., Int. Rev. Exp. Pathol. 4:127-157 (1965)). In contrast, apoptosis is usually associated with physiological conditions, e.g., embryogenesis (Bellaris, J. Anat. 95:54-60 (1961); Saunders, Science 154:604-612 (1966)) and metamorphosis (Truman, Ann. Rev. Neurosci. 7:171-188 (1984)). Interestingly, morphological features of physiological cell death in C. elegans resemble, in general, those of apoptosis in vertebrates (Ellis et al., Ann. Rev. Cell Biol. 7:663-698 (1991)). However, deviations from the standard descriptions of necrosis and apoptosis are often observed. It is uncertain whether this morphological classification reflects real differences in underlying mechanisms of cell death.

Although the initiation and morphology of cell death vary, there is evidence which suggests that most

-5-

physiological and some pathological cell deaths may share a common feature involving the activation of cell death genes. The existence of a genetic cell death program in a variety of organisms is suggested by the observation that protein and RNA synthesis inhibitors can prevent or delay a variety of cell deaths (insect metamorphosis, prostate regression, vertebrate neuronal cell death and thymocyte cell death) (Lockshin, J. Insect Physiol. 15:1505-1516 (1969); Stanisic et al., Invest. Urol. 16:19-22 (1978); Martin et al. (1988) supra; Oppenheim and Prevette (1988) supra; Cohen and Duke (1984) supra). New RNA and protein species have been found after the initiation of cell death in the rat prostate after castration (Buttayan et al., Molecular Endocrinology 2:650-657 (1988); Lee et al., Prostate 7:171-185 (1985)). Thus, a better understanding of the mechanisms of cell death would have wide biological application and provide a basis for altering or controlling the process.

## 20 Summary of the Invention

The present invention relates to genes, referred to herein as cell death-protective genes, which function to protect cells against programmed cell death by antagonizing the activity of genes which cause cell death. As described herein, Applicants have identified what appears to be a key or master regulatory gene whose activity determines whether a cell survives or undergoes cell death.

Specifically, a cell death-protective gene from the nematode Caenorhabditis elegans, called ced-9, has been identified, sequenced, and characterized. ced-9 is essential for C. elegans development and apparently functions by protecting cells which normally live during development from programmed cell death. As is also

-6-

described herein, a mutation that constitutively activates ced-9 prevents cells which normally die during development from undergoing programmed cell death, and mutations that inactivate ced-9 result in the deaths of cells which normally survive during development and consequently, in embryo lethality. ced-9 has been shown to function by antagonizing the activities of the cell death genes ced-3 and ced-4. Thus, the C. elegans ced-9 gene appears to act as a binary switch to regulate programmed cell death. Results described herein indicate that many and possibly all cells that survive during C. elegans development do so because ced-9 activity prevents them from undergoing programmed cell death.

15 In addition, a human equivalent of the C. elegans ced-9 gene has been discovered. The deduced amino acid sequence of the ced-9 gene product was found to have about 23% identity and about 47% similarity to the product of the human oncogene bcl-2. This structural similarity, together with previous studies on bcl-2 activity in lymphocytes, strongly suggests that bcl-2 is a human equivalent of ced-9. Applicants further provide methods for identifying other cell death-protective genes from a variety of organisms, including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast), and possibly plants. Furthermore, comparison of ced-9, bcl-2, and other cell death-protective genes and their encoded products provides a way to define key functional features or regions of these genes and gene products. Those features or parts that are conserved between these genes or their gene products are most likely to be functionally important.

Applicants further provide methods and agents for altering the occurrence of cell death in a population of



-7-

cells and hence, affecting the proliferative capacity and longevity of tissues or organisms. Methods and agents for both decreasing and increasing cell deaths are described. The agents may be all or portions of the

5 cell death-protective genes and encoded products, or derivatives, mimetics, activators or inactivators, or agonists or antagonists of the activity of cell death-protecting genes.

As a result of this work, methods and agents for

10 altering cell death are available for therapeutic or preventive treatment of diseases or conditions involving cell death. Methods and agents for reducing cell death are available and are potentially useful for treating disorders and conditions, including those associated

15 with aging, hair loss, stroke, traumatic brain injury, myocardial infarction, degenerative diseases (including Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and Duchenne's muscular dystrophy) and viral and other types of

20 infection (such as with the human immunodeficiency virus or HIV). Methods and agents for increasing cell deaths are also available which are potentially useful for decreasing the growth of or for killing specific cell populations, such as infected cells or autoreactive

25 immune cells. These methods and agents may also be useful for treating diseases or conditions characterized by excessive cell growth or an abnormally low frequency of cell death (e.g., neoplasia and other cancerous growth). Methods and agents which increase cell death

30 are also potentially useful for treating viral, parasitic, and other infections and to kill undesirable organisms, for example, in pest control or biological containment applications.

-8-

### Brief Description of the Drawings

Figure 1 is a diagrammatic representation of a model for ced-9 function. (a) ced-9 is a negative regulator of ced-3 and ced-4 activity. (b) ced-9 acts as a binary switch to regulate programmed cell death. When ced-9 is active, the activities of ced-3 and ced-4 are blocked, and the cell survives. When ced-9 is inactive, ced-3 and ced-4 are activated, leading to programmed cell death.

10 Figures 2A-J show the nucleotide sequence (SEQ ID NO:1) of the genomic region containing the C. elegans ced-9 gene, with selected restriction sites.

Figure 3 shows the nucleotide sequence (SEQ ID NO:2) of a particular ced-9 cDNA, with selected  
15 restriction sites and the predicted translation product.

Figure 4 shows the predicted amino acid sequence (SEQ ID NO:3) of the Ced-9 protein as deduced from the genomic and cDNA sequences.

Figure 5 shows changes observed in several ced-9 mutants. Shown are changes in the DNA sequence and the resulting predicted change in the protein sequence associated with each mutation.

Figure 6 shows the optimized alignment of the C. elegans Ced-9 (SEQ ID NO:3) and human Bcl-2 (SEQ ID NO:5) proteins. Identical residues are indicated by vertical bars between the sequences, and similar residues are indicated by one or two dots (. or :), for weak and strong similarity, respectively. A residue that is mutated in the gain-of-function allele n1950 is conserved and has been boxed. Residues mutated in the loss-of-function alleles, n1653ts and n2077, are also indicated by boxes.

Figures 7A-E show the cDNA sequence of bcl-2 (SEQ ID NO:4). The coding sequence is from nucleotides 1459  
35 to 2178, inclusive.

-9-

Figure 8 shows the screen for mutations that result in a loss of ced-9 function.

Figure 9 is a diagram of the ced-9 cloning strategy and cosmid rescue. a) Genetic map of the ced-9 region. Relevant genes as well as the approximate position of the N2/RC301 restriction fragment length polymorphisms (RFLPs) used to map ced-9 are shown. b) Number of recombination events observed between various markers in the unc-69 to unc-49 interval. The nP55 polymorphism did not separate from unc-69 in these experiments, suggesting that unc-69 is to the right, or close and to the left of nP55. c) Cosmid rescue of unc-69 and ced-9. Cosmids situated between the nP55 and nP56 RFLPs (recognized by cosmids C15B3 and C38H2, respectively) were injected into unc-69 or unc-69 ced-9/++ animals, and established transgenic lines were tested for rescue of the unc-69 and ced-9 phenotypes. ND: not determined.

Figure 10 shows the ced-9 locus. a) Restriction map. b) Rescue ability of deletions and point mutations in the locus. c) ced-9 transcripts and location of introns and exons.

#### Detailed Description of the Invention

Programmed cell death is cell death which occurs during normal development and/or which involves the activities of cell death genes, some of which may be suicide genes. Programmed cell death is a fundamental aspect of normal development in invertebrates and vertebrates and of vertebrate tissue homeostasis, and may also be an underlying pathological mechanism in disorders which involve cell death, including degenerative diseases, hair loss, stroke, traumatic brain injury, and myocardial infarction, conditions associated with aging, and viral and other types of

infection. In addition, some cancers are believed to be caused in part by defects in cell death processes.

This invention relates to genes, referred to as cell death-protective genes, which protect cells against programmed cell death by antagonizing the activities of cell death genes (i.e., genes whose activity cause cell death). As described below, a cell death-protective gene from the nematode Caenorhabditis elegans, called ced-9, has been identified, sequenced, and characterized. Mutations which constitutively activate and inactivate ced-9 gene function have been identified and are also described below. As further described below, the deduced amino acid sequence of the ced-9 gene product (SEQ ID NO:3) was found to have about 23% identity and about 47% similarity to the product of the human oncogene bcl-2 (SEQ ID NO:5). The structural and functional similarity of bcl-2 to ced-9 strongly suggests that bcl-2 may be a human equivalent of ced-9 and thus, a cell death-protective gene. Using ced-9 and bcl-2, other cell death-protective genes from a variety of organisms can be obtained. In addition, comparison of equivalent genes and their encoded products, as well as mutational analysis, is expected to indicate key functional features or regions of the genes or gene products. The cell death-protective genes and their gene products are further useful for developing and identifying agents which affect the activity of cell death-protective genes. These agents may be useful for altering (increasing or decreasing) the occurrence of cell death in a cell population or organism, and thus, altering the longevity of the cell population or organism. Further described below are bioassays which are useful for testing and screening for novel cell death-protective genes, mutations in these genes and

-11-

agents which affect the activity of the genes. Other uses of the invention are also described.

The activity of a cell death-protective gene refers herein to the activity of the encoded product(s) of the gene as well as to the gene per se. Thus, agents and mutations which affect the activity of a cell death-protective gene include those which affect the activity of the gene or a product of the gene. The agents may interact with the gene or RNA or protein encoded by the gene, or may exert its effect more indirectly. The agents may affect the level of expression as well as the function of the gene or gene product.

#### Genetic Analyses of the ced-9 Gene

A cell-death protective gene, called ced-9, has been identified in the nematode C. elegans that functions to prevent cells which normally live during development from undergoing programmed cell death. The ced-9 gene was defined by a dominant gain-of-function (gf) mutation, called n1950, which was mapped to chromosome III. The n1950 mutation constitutively activates the ced-9 gene and causes cells which normally die during development to live. Activated ced-9 prevents programmed cell deaths throughout the animal, and, as shown for certain nerve cells, not only prevents cells from dying, but also generates surviving cells that are sufficiently healthy to function. ced-9(n1950) also shows a maternal effect, suggesting that the maternal ced-9 gene product is contributed to the developing oocyte. Genetic analysis of ced-9(n1950) is further described in Example 1 and Table 1 (tables are at the end of the Detailed Description).

Loss-of-function (lf) mutations which inactivate the protective function of ced-9 and cause cells which normally live during C. elegans development to die were

also identified. These mutations result in embryonic lethality in the progeny of homozygous animals, indicating that ced-9 function is essential for development. Four ced-9(lf) mutations were isolated, nDf40, n2077,  
5 n2161, and n1653ts. The lf mutations also show maternal effects. The amount of wild-type ced-9 product contributed by heterozygous mothers to homozygous ced-9(lf) embryos seems to be sufficient to allow these embryos to survive and develop almost normally. As a  
10 consequence of this maternal rescue, the lethality that results from an absence of ced-9 function during early development is apparent only in the second generation. Most of the ectopic cell deaths observed in the first generation of homozygous ced-9(lf) animals occur late,  
15 during post-embryonic development. It is possible that these late lineages are more seriously affected because dilution or degradation has reduced the amount of maternal ced-9 product to a level at which it cannot effectively protect against cell death. The isolation  
20 and genetic analysis of these loss-of-function mutations are further described in Examples 2 and 3 and Table 2.

As described in Example 4, the ced-9 gene appears to prevent cell death by antagonizing the activities of the cell death genes, ced-3 and ced-4, which have been  
25 shown to be required for almost all programmed cell deaths which occur in the development of C. elegans (Ellis and Horvitz, Cell 44:817-829 (1986)).

These results indicate that ced-9 acts as a binary switch to regulate programmed cell death (Figure 1).  
30 Remarkably, it seems that many and possibly all cells that survive during C. elegans development do so because ced-9 gene activity prevents them from undergoing programmed cell death. Furthermore, cells protected by a constitutively activated ced-9 gene appear to be  
35 healthy and to function normally. Thus, ced-9 seems to

-13-

be a key or master regulatory gene of cell death processes.

#### Sequence Analysis of the *ced-9* Gene and Product

The genomic region containing the *ced-9* gene was  
5 cloned and sequenced, as described in Example 5. Figure  
2 shows the nucleotide sequence (SEQ ID NO:1) of this  
region, including the location of selected restriction  
sites.

Several *ced-9* cDNAs representing the same or  
10 different transcripts were obtained and sequenced, as  
described in Example 5. The nucleotide sequence (SEQ ID  
NO:2) of one of these cDNAs is shown in Figure 3 with  
restriction sites and the amino acid sequence (SEQ ID  
NO:3) of the predicted translation product. As shown in  
15 Figure 4, *ced-9* encodes a 280 amino acid (aa)  
polypeptide.

The gain-of-function mutation, *n1950*, was also  
sequenced. As shown in Figure 5, the *n1950* mutation,  
which is responsible for the gain-of-function change in  
20 *ced-9* activity, is associated with a glycine to glutamic  
acid change at codon 169. It is likely that this amino  
acid alteration is the consequence of the *n1950* mutation  
and thus is functionally responsible for the increased  
activity of *ced-9*. However, although no other  
25 alterations in *ced-9* are known to be present in *n1950*  
mutant strains, it remains possible that another  
alteration exists and that it is this other alteration  
that is responsible for the gain-of-function change in  
*ced-9* activity. If so, this other amino acid alteration  
30 is nonetheless defined by the *n1950* mutation and its  
molecular identity can be determined by DNA sequencing,  
using established methods. The functional importance of  
DNA sequence alterations associated with *ced-9* mutations  
can be verified in transgenic *C. elegans* animals which

carry the sequence alteration alone. DNA containing alterations in the wild-type gene can be made by standard methods of in vitro mutagenesis and used to construct the transgenic animals.

- 5        The loss-of-function mutations, n1653ts and n2077, were also sequenced and found to be associated with a tyrosine to asparagine change at codon 149 and a glutamine to premature termination at codon 160, respectively.

10    Similarity Between ced-9 and a Human Oncogene

- Sequence similarity to the ced-9 gene product (SEQ ID NO:3) was discovered in the product of the human oncogene bcl-2 (SEQ ID NO:5) (Tsujimoto et al., Proc. Natl. Acad. Sci. USA 83:5214-5218 (1986)). Alignment of  
15    the two sequences shows 23% identity and 47% similarity between the two proteins (Figure 6). Alignment of the two sequences was generated with the Gap program in the Sequence Analysis Software Package (Genetics Computer Group, Wisconsin), which uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443-453 (1970)) to find the  
20    alignment of two complete sequences that maximize the number of matches and minimizes the number of gaps.

- bcl-2 is one of a number of genes, of both viral and cellular origin, which are thought to be inhibitors  
25    of cell death processes (Vaux et al., Nature 335:440-442 (1988); Henderson et al., Cell 65:1107-1115 (1991); Ciem et al., Science 254:1388-1390 (1991)). Overexpression of bcl-2 prevents or delays the onset of apoptic cell death in both B cells and T cells (Vaux et al. (1988)  
30    supra; Nunez et al., J. Immun. 144:3602-3610 (1990); Sentman et al., Cell 67:879-888 (1991); Strasser et al., Cell 67:889-899 (1991)). These cell deaths seem to involve the activities of cell death genes, as gene expression is required for the process (Duke and Cohen,



-15-

Lymphokine Res. 5:289-299 (1986)). In many tissues in which homeostasis is regulated by cell death, bcl-2 expression occurs in progenitor and long-lived cells (Hockenbery et al., Proc. Natl. Acad. Sci. USA 88:6961-5 6965 (1991)). The structural similarity of the Bcl-2 protein to Ced-9, together with these previous findings on bcl-2 activity in lymphocytes, suggest that, like ced-9, the bcl-2 gene antagonizes the activities of cell death genes and is required in cells that survive to 10 protect them from programmed cell death.

#### Cell Death-Protective Genes in Other Organisms

As a result of the work described herein, a gene which plays a key role in determining cell death has been identified, sequenced and characterized. This 15 invention makes it possible to identify and isolate equivalent genes in other organisms, including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast), and possibly plants. The reasonableness of this 20 approach has been demonstrated by the structural and functional similarity of the human gene bcl-2 to ced-9. As discussed below, there is evidence to suggest that programmed cell death is important in the development of a variety of organisms and tissues, and that different 25 types of cell deaths, whether physiological or pathological, may share a common mechanism.

Evidence suggests that cell deaths that are mechanistically similar to the programmed cell deaths which occur in the development of C. elegans and other 30 invertebrates may be very common in vertebrate development, as well. First, cell deaths that are similar to the programmed cell death seen in invertebrates were also observed in vertebrates (Glucksmann (1951) supra; Saunders and Fallon, in: Major

Problems in Developmental Biology (25th Symposium of the Society for Developmental Biology), Lockes (ed.)

Academic Press, New York, 1966, pp. 289-314; Carr and Simpson, Dev. Brain Res. 2:57-162 (1982)). Some of

5 these cells die shortly after they are born without obvious differentiation and others have been shown to be determined to die days before death occurs.

Second, some vertebrate neuronal cell deaths involve cell interactions and have been thought by some  
10 to be different from the apparently cell autonomous programmed cell deaths observed in invertebrates. However, even among the cell dependent deaths of these vertebrate neurons, the expression of new genetic information was found to be necessary for cell death, suggesting that a  
15 cell death program must be activated. Thus, it is possible that genes similar to those responsible for programmed cell death in C. elegans (ced-3 and ced-4) act in these vertebrate cell deaths as well.

Third, although many cell deaths in invertebrates  
20 are invariant and many in vertebrates are variable, the same mechanisms may nonetheless be responsible. Specifically, invariability does not reveal the mechanism of cell death. For example, invariability could be the result of an invariant cell-cell  
25 interaction. Thus, the linker cell in C. elegans males always dies at a certain specific time, but its death requires cell-cell interaction. The invariable programmed cell deaths in moths can, in fact, be prevented by manipulating their hormonal environment or  
30 changing cell-cell interactions. These observations argue that the distinction between programmed cell death during invertebrate development and cell death during vertebrate development may be more superficial than real. All of these cell deaths are influenced by  
35 genetic factors. Such argument leads to the notion that

-17-

many and possibly all naturally occurring cell death during development may involve similar mechanisms.

The induction of specific genes has been found during the cell death processes in vertebrates. The  
5 induction of TRPM-2 was observed during a variety of cell death processes in rodents (Buttayan et al., Mol. Cell. Biol. 9:3473-3481 (1989)). TRPM-2 RNA is at high levels in cells that die during prostate regression in the adult male rat after castration, during renal  
10 atrophy following ureteral obstruction in rat, during necrosis of interdigital tissues of the mouse limb bud, and during the chemotherapeutic regression of tumors in rat. Thymocyte cell deaths induced in response to a variety of stimuli can have very similar morphological  
15 and biochemical properties, implying the involvement of a single mechanism of cell death. These observations suggest strongly that many types of cell deaths may share a similar mechanism.

In addition, it is possible that of the many human  
20 disorders characterized by extensive cell deaths, such as degenerative diseases, stroke, myocardial infarction and traumatic brain injury (for example, see Choi, Neuron 1:623-634 (1988)), some are caused by processes that inactivate or bypass the functions of genes like  
25 bcl-2 and ced-9. Furthermore, intracellular calcium has been implicated as a common mediator in a variety of pathological cell deaths, including deaths caused by external injury, toxins, degenerative diseases, ischemia, and hypoxia (Schanne et al., Science 206:700-  
30 702 (1979); Farber, Life Science 29:1289-1295 (1981)). Interestingly, amino acid sequence analysis of the C. elegans ced-4 gene product indicates that the Ced-4 protein may contain calcium-binding domains (Yuan, Ph.D. thesis, Harvard University, 1989, p. 139).

### Uses of the Invention

This invention provides agents and methods based on ced-9, bcl-2, and other cell death-protective genes that are useful for diagnosis and treatment (both therapeutic  
5 and preventive) of a variety of disorders and conditions involving cell death. The invention is applicable to a variety of organisms, including humans. The genes and their encoded products can be used directly in therapeutics or provide a basis for designing and  
10 identifying agents which affect the occurrence of cell death. In addition, mutant forms of these regulatory genes, their encoded products and derivatives of the encoded proteins are available that are potentially useful for treatment.

15 Other cell death-protective genes can be obtained using the methods provided by this invention. As discussed above, it is likely that genes that are structurally and functionally similar to the C. elegans ced-9 gene function in a variety of organisms, including  
20 vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast), and possibly plants. These equivalent genes have nucleotide sequences similar to portions of the ced-9 gene, or their encoded products have amino acid  
25 sequences similar to portions of the ced-9 protein. Equivalent genes also have similar activity to ced-9, in that they protect the cells in which they function from cell death. For example, the human gene bcl-2 was found to be equivalent to ced-9, as described herein.

30 Novel cell death-protective genes can be identified by any number of detection methods which utilize a defined nucleotide or amino acid sequence or antibodies as a probe. The genomic (SEQ ID NO:1) and cDNA (SEQ ID NO:2) nucleotide sequences of ced-9 and the deduced  
35 amino acid sequence (SEQ ID NO:3) of the Ced-9 protein

-19-

are shown in Figures 2, 3, and 4, respectively. The bcl-2 gene and gene products can also be used as probes for cell death-protective genes. The cDNA nucleotide sequence of bcl-2 (SEQ ID NO:4) and the deduced amino acid sequence (SEQ ID NO:5) of the Bcl-2 protein are shown in Figures 6 and 7. For example, nucleic acid (DNA or RNA) containing all or part of the ced-9 or bcl-2 genes can be used as hybridization probes or as polymerase chain reaction (PCR) primers. Degenerate oligonucleotides derived from the amino acid sequences of the ced-9 or bcl-2 gene products can be used in these methods. In addition, antibodies, both polyclonal and monoclonal, which bind specifically to the Ced-9 and/or Bcl-2 protein can be produced and used as immunoprobes to screen expression libraries of genes. Databases containing known molecular (nucleotide or amino acid) sequences can also be searched for molecules which are structurally similar to ced-9, bcl-2, or their encoded products.

One strategy for detecting novel cell death-protective genes in various organisms is to initially probe animals which are taxonomically closely related to the source of the probes, for example, probing other worms with a probe derived from ced-9 or probing other mammals with a probe derived from bcl-2. Closely related species are more likely to possess cell death-protective genes or products which are detected with the probe than are more distantly related organisms. These new genes then provide additional sequences with which to probe the molecules of other animals, some of which may share conserved regions with the new genes or gene products but not with ced-9 or bcl-2. This strategy of using related genes in taxonomically closer organisms as stepping stones to genes in more distantly related organisms can be referred to as walking along the

taxonomic ladder. However, cell death-protective genes or gene products from a variety of organisms may possess considerable sequence similarity and hence, be identifiable by more direct approaches.

5       The ced-9 and bcl-2 gene products were found to have 23% identity and 47% similarity. The molecular similarity between the ced-9 and bcl-2 gene products is useful, because the similarities between the two proteins reveal which parts or features of these  
10 molecules are important for function. For example, an activated bcl-2 may be produced by mutation of the codon which is equivalent to the site of the n1950 mutation in ced-9. More insights on the structure-function relationship of cell death-protective genes are expected  
15 to be obtained as more genes equivalent to ced-9 and/or bcl-2 are compared. This knowledge can be used to develop novel molecules which mimic or alter the activity of ced-9, bcl-2 or other cell death-protective genes.

20       Cell death-protective genes identified as described above can be sequenced by standard methods. Mutated forms of the genes may be identified by such methods, and some of these mutations are expected to constitutively activate and some to inactivate the genes  
25 like the n1950 gain-of-function and the loss-of-function mutations in ced-9. Mutationally activated and inactivated forms of cell death-protective genes may be useful for treatment of various disorders, as described further below. In addition, mutagenesis and other sorts of  
30 alterations can be performed on the genes and their encoded products to obtain other activated or inactivated proteins.

      Mutations may also produce cell death-protective proteins with novel properties. For example, it is conceivable that a cell death-protective gene could be al-  
35

-21-

tered such that the gene product actively kills cells, rather than protecting them from cell death, perhaps by activating cell death genes or interfering with the function of wild-type cell death-protective gene products. Mutations and other alterations can be accomplished using known methods, such as in vivo and in vitro mutagenesis.

Furthermore, ced-9, bcl-2, or other cell death-protective genes, the corresponding mutant genes, and encoded products can be used to develop agents that activate or inactivate or modulate the activity of the cell death-protective genes. The source of the agents can be such traditional sources as extracts (e.g., bacterial, fungal or plant) and compound libraries, or can be provided by newer methods of rationale drug design. Information on functionally important regions of the genes or gene products, gained by sequence and/or mutational analysis, as described above, would be useful in drug design. The activity of the agents can be verified both by in vivo tests on wild-type, mutant, or transgenic animals containing various forms of ced-9, bcl-2, or other cell death-protective genes, as described below, and by in vitro tests using either cells expressing such genes or the products of these genes directly in biochemical experiments. Potential agents may include all or portions of the ced-9 or bcl-2 genes or gene products (RNA, protein), all or portions of other cell death-protective genes and their encoded products, nucleic acid or peptide derivatives of cell death-protective genes and gene products (e.g., smaller polypeptides and peptides), as well as peptido-mimetics, and other molecules which mimic or affect the activity of cell death-protective genes. The agents can also be portions or derivatives of genes which do not by

themselves protect cells from programmed cell death but which interact with cell death-protective genes.

This invention further provides bioassays which measure the activity of cell death-protective genes, and  
5 hence, are useful for identifying cell death-protective genes, for testing mutations in these genes, and for developing agents which mimic or alter cell death-protective activity. The bioassays can be further used to screen expression gene libraries for novel cell  
10 death-protective genes from nematodes and other organisms.

In one bioassay, genes or agents are introduced into nematodes to test their effect on cell deaths in vivo. Wild-type, mutant, or transgenic nematodes can be  
15 used as appropriate for the expected effect being tested. In one embodiment of the bioassay, transgenic nematodes are produced using sample DNA containing a candidate cell death-protective gene, a mutant cell death-protective gene or a gene library, to observe the  
20 effect of the sample DNA on the pattern of cell deaths during development of the nematode, using the methods of genetic analysis described for the ced-9 mutations. For example, a candidate gene can be introduced into a nematode which has a loss-of-function mutation in ced-9  
25 to produce a transgenic nematode. A decrease in cell deaths compared to nontransgenic nematodes would indicate that the sample gene has cell death-protective activity. Similarly, a mutant cell death-protective gene which is inactivated would fail to complement the  
30 ced-9 loss-of-function mutation in the transgenic nematode, whereas a constitutively activated gene would decrease the number of cell deaths resulting from the mutation.

In another embodiment of the nematode bioassay,  
35 wild-type, mutant, and transgenic nematodes are used to



-23-

test the effects of specific peptides and other small molecules in order to identify drugs that mimic, increase or decrease cell deaths. For example, wild-type animals can be used to test agents that inactivate or decrease the activity of ced-9 and cause increased cell deaths, or that activate or increase the activity of ced-9 and decrease or prevent cell deaths. Mutant or transgenic animals in which ced-9 is underexpressed or inactivated could be used to identify agents that mimic ced-9 in preventing cell death or which act as agonists of cell death-protective activity. Likewise, mutant or transgenic animals in which ced-9 is overexpressed or constitutively activated can be used to identify agents which act as antagonists of cell death-protective activity. Nematodes expressing wild-type ced-9 could be used to identify agents which activate or inactivate the ced-9 gene. The agents may include genes which are not cell death-protective genes but which interact with, regulate, or otherwise affect the activity of ced-9. The agents can be introduced into nematodes by microinjection, diffusion, or ingestion.

Furthermore, agents which affect the activity of other cell death-protective genes, such as bcl-2, can be tested by transgenic animals with a loss-of-function mutation in ced-9. Agents which are non-cell death-protective genes can be tested on cell death-protective genes other than ced-9 by constructing doubly transgenic animals. These animals can be made by crossing a transgenic line which expresses a cell death-protective gene and an inactivated ced-9 gene with a transgenic line which expresses the agent gene.

An in vitro bioassay is also provided. In this bioassay, cultured mammalian cells are used to test genes and agents. Expression gene libraries can also be screened by this method. For example, genes, including

genes which are structurally similar to ced-9 or bcl-2, can be introduced into mammalian cells by standard transfection methods to see if they protect from cell death under conditions which induce cell death, such as exposure to toxins or infection by yeast or bacteria. Mutations which activate or inactivate or otherwise affect cell death-protective activity can be tested. Furthermore, transfected mammalian cells which express a wild-type or mutant cell death-protective gene can be used to test agents which increase or decrease the activity of cell death-protective genes.

Using the above-described nucleic acid and antibody probes and bioassays, the identification and expression of ced-9, bcl-2 or other cell death-protective genes in cultured cells, tissues, and whole organisms can be studied to gain insights into their role in development and pathology. For example, these methods of detection and bioassay can be used to determine if certain mutations in cell death-protective genes, such as bcl-2, are associated with a pathological condition, such as a degenerative disorder.

This invention further provides means of altering or controlling the activity of a cell death-protective gene in a cell or organism, and, thus, to affect the occurrence of cell death. Activity of the regulatory gene can be altered to either increase or decrease cells deaths in a population of cells and, thus, affect the proliferative capacity and/or longevity of a cell population, organ, or entire organism.

ced-9, bcl-2, or other cell death-protective genes, and related and derivative products can be used to protect against cell death of any sort, including degenerative disease, stroke, traumatic brain injury, myocardial infarction, and viral and other types of infection, as well as cell death associated with normal

-25-

aging. The gene, its encoded RNA, the protein encoded by the gene, or a peptide derived from or related to the gene can be delivered to the affected cells by various methods appropriate for the cells or organs being  
5 treated, including gene therapy. A non-peptide molecule which mimics, activates, or enhances the activity of a protein encoded by ced-9 or other cell death-protective gene, or polypeptide or peptide derivative, and which is designed on the basis of knowledge of the encoded  
10 protein, can also be used. That is, the gene or its product may be used either directly to protect against cell death or as the basis for developing another agent that can function like or increase the activity of the gene or its encoded product.

15        Mutationally activated forms of the genes can also be used to protect against cell death. Again, the mutated gene, its encoded RNA, the mutant protein encoded by the gene, a peptide derived from or related to the mutant protein, or a non-peptide mimetic,  
20 activator or agonist can be used. The n1950 mutation in ced-9 defines one way to make such a gene activated. A mutation equivalent to n1950 can be placed in a cell death-protective gene similar to ced-9 to activate it. For example, a constitutively activated bcl-2 protein  
25 might be produced by making a glycine to glutamic acid change at codon 145, as shown in Figure 6, or other sequence alteration equivalent to the one which is responsible for the phenotype of the n1950 mutation in ced-9. (It has not yet been definitively shown that the  
30 glycine to glutamic acid alteration of codon 169 of ced-9 is responsible for the activated phenotype of the n1950 mutation. If it is not, the other mutational change(s) in ced-9 responsible for the activation of this gene can be identified as described above and  
35 produced in bcl-2 by in vitro mutagenesis to activate

-26-

bcl-2). The mutant Bcl-2 protein may then be used as a clinically useful molecule or as a basis for developing or identifying a clinically useful molecule which protects from cell death.

- 5           Alternatively, ced-9, bcl-2, or other cell death-protective genes and their encoded products can be inactivated, or their activity reduced, in order to increase the frequency of cell death. This would be useful, for treating diseases and conditions
- 10 characterized by an abnormally low frequency of cell death or excessive cell growth, such as neoplastic growth and other cancers. Interestingly, the human cell death-protective gene bcl-2 is also an oncogene, suggesting that cell death processes can be affected in
- 15 neoplasia. Methods and agents which increase cell death would also be useful for decreasing the growth of or eliminating specific cell populations. For example, populations of autoreactive immune cells may be eliminated or reduced for treating autoimmune disorders.
- 20   The activity of bcl-2 or other equivalent cell death-protective gene may be inactivated by using single stranded nucleic acid having an antisense sequence which is complementary to the normal transcript of the cell death-protective gene, such as antisense RNA, or DNAs
- 25 which encode the antisense nucleic acid, or inactivators or antagonists of cell death-protective activity. These agents can be delivered by a variety of methods, including gene therapy. Inactivation of cell death-protective genes may also be useful in treating viral,
- 30 parasitic and other types of infection, such as human immuno-deficiency virus (HIV) infection. A recombinant gene encoding an inactivator or antagonist of cell death-protective activity, such as antisense RNA which is complementary to the transcript of a cell death-
- 35 protective gene, may be linked to a viral promoter which

-27-

is specifically activated by a viral protein. The recombinant gene is introduced into infected cells. Infected cells containing the viral protein would then be killed and uninfected cells would be unaffected.

5        Inactivation of cell death-protective genes may also be used to kill organisms for the purpose of biological containment, pest control, or other applications in which populations of undesirable organisms are to be reduced. For example, suicide genes  
10        used for biological containment of recombinant bacteria have been reported (Genetic Engineering News, Nov. 1991, p. 13). The suicide genes were engineered to be expressed simultaneously with the desired recombinant gene product so that the recombinant bacteria die upon  
15        completion of their task. The present invention provides for construction of recombinant suicide genes encoding antisense RNAs or other inactivators or antagonists of ced-9 or other cell death-protective genes which are useful in organisms in addition to  
20        bacteria, for example, in insects, fungi, and transgenic rodents.

Agents which inactivate or inhibit cell death-protective genes can further be used for pest control. For example, many nematodes are human, animal, or plant  
25        parasites. Populations of such parasites could be reduced or eliminated by causing their cells to undergo programmed cell death. Parasites present in host animals, including humans, may also be reduced by treatment with agents, such as antisense RNAs, which  
30        decrease the activity of a cell death-protective gene specific to the parasite and which leave the host animal unharmed.

The following examples illustrate the invention and are not intended to be limiting in any way.

EXAMPLE 1

Gain-of-Function Mutation in ced-9

While screening for new C. elegans mutations that affect programmed cell death (Ellis and Horvitz, Development 112:591-603 (1991)), a dominant mutation, n1950, was isolated and genetically characterized, that prevents programmed cell deaths. n1950 was mapped to the right arm of the third chromosome, close to and about 0.05 map units to the right of the mutation unc-69(e587). The n1950 mutation defines a new gene, ced-9 III.

To quantify the effects of the ced-9(n1950) mutation on programmed cell deaths, cells in the anterior half of the pharynx of ced-9(n1950) animals were counted. In wild-type animals there are 49 cell nuclei in this region (Sulston et al., Devl. Biol. 100:64-119 (1983); Albertson and Thomson, Phil. Trans. R. Soc. B275:299-325 (1976)), and in ced-3 and ced-4 animals there are 12-14 additional nuclei (Table 1a). Similarly, in ced-9(n1950) animals there are about 13 extra nuclei in the anterior pharynx. These extra nuclei correspond exactly in position as well as in number to those that fail to die in ced-3 and ced-4 mutants.

Many extra cells survive not only in ced-9(n1950) homozygotes but also in ced-9(n1950)/+ heterozygotes, indicating that the n1950 phenotype is dominant (Table 1b). In addition, the ced-9(n1950) mutation has a maternal effect: about twice as many cells fail to die in heterozygotes generated by mothers carrying at least one copy of the ced-9(n1950) mutation than in heterozygotes generated by homozygous wild-type mothers (Table 1b), suggesting that maternal ced-9 gene product is contributed to the developing oocyte.

-29-

Two observations indicate that ced-9(n1950) is a gain-of-function (gf) mutation. First, n1950 is a rare mutation with dominant effects (only one allele was recovered in a screen of over 24,000 haploid genomes  
5 (Ellis and Horvitz (1991) supra), which is a frequency about 10-fold lower than that at which typical loss-of-function mutations are recovered (Brenner, Genetics 77:71-94 (1974); Meneely and Herman, Genetics 92:99-115 (1979); Greenwald and Horvitz, Genetics 96:147-164  
10 (1980)). Second, a deletion (nDf40) that removes the ced-9 gene does not have a dominant effect on cell death (Table 1b).

To study the effects of ced-9(n1950) on programmed cell deaths in regions other than the anterior pharynx,  
15 it was determined whether n1950 could prevent the accumulation of cell corpses in ced-1 and ced-5 mutants. In wild-type animals, dying cells are rapidly engulfed and degraded by a neighboring cell. In ced-1 and ced-5 mutants, this engulfment process is blocked, leading to  
20 an accumulation of undegraded cell corpses that can easily be seen in young larvae (Hedgecock et al., Science 220:1277-1279 (1983); Ellis et al. Genetics 129:79-94 (1991)). Mutations that inactivate ced-3 or ced-4 block programmed cell death and therefore prevent  
25 the accumulation of dead cells in ced-1 or ced-5 animals (Ellis and Horvitz, Cell 44:817-829 (1986)). Similarly, very few corpses appear anywhere in ced-1; ced-9(n1950) or ced-9(n1950); ced-5 double mutants (Table 1c). Thus, the ced-9(n1950) mutation, like mutations in ced-3 and  
30 ced-4, prevents programmed cell deaths throughout the animal.

The effects of ced-9(n1950) on the survival and function of a specific pair of nerve cells, the HSNs (hermaphrodite-specific neurons) were also studied  
35 (Trent et al., Genetics 104:619-647 (1983); White et

al., Phil. Trans. R. Soc. B311:1-340 (1986); Desai et al., Nature 336:638-646 (1988); Desai and Horvitz, Genetics 121:703-721 (1989)). The two HSN neurons innervate the vulval muscles and control egg-laying by hermaphrodites. Mutations in the gene eql-1 cause these cells to undergo programmed cell death, resulting in egg-laying defective animals (Ellis and Horvitz, Cell 44:817-829 (1986); Trent et al., (1983) supra; Desai and Horvitz (1989) supra). Mutations in ced-3 and ced-4, which block programmed cell death, prevent the HSNs from dying in eql-1 mutants and suppress the egg-laying defect (Ellis and Horvitz (1986) supra). Similarly, the HSNs are present in ced-9(n1950); eql-1 double mutants, and egg-laying by these animals is normal (Table 1d). Thus, ced-9(n1950), like the ced-3 and ced-4 mutations, not only prevents cells from dying but, at least in this case, also generates surviving cells that are sufficiently healthy to function.

#### Methods

The data presented in Table 1 were obtained as follows. Cell survival was quantified by counting the cells in the procorpus and metacarpus, which together constitute the anterior half of the pharynx (Albertson and Thomson, Phil. Trans. R. Soc. B275:299-325 (1976)). In wild-type animals there are 49 cell nuclei in this region. Cells that die are generated in characteristic positions (Sulston et al., Devl. Biol. 100:64-119 (1983)), making it easy to identify and count cells that have failed to die. The genotypes of animals studied for Table 1a were as shown.

The complete genotypes of the animals studied for Table 1b were, from top to bottom: wild-type (N2), non-Unc progeny of eT1 unc-36/nDf40 dpy-18 males crossed with unc-36 hermaphrodites, non-Unc progeny of n1950



-31-

males crossed with unc-69 hermaphrodites, Unc Dpy progeny from n1950/unc-69 dpy-18 hermaphrodites, non-Lon non-Dpy progeny from dpy-17 lon-1/n1950 dpy-18 hermaphrodites, Unc-49 progeny from unc-69/n1950 unc-49 heterozygous hermaphrodites, non-Unc progeny of wild-type (N2) males crossed with unc-69 n1950 hermaphrodites, and n1950 self-progeny from n1950 homozygous hermaphrodites.

For the pharyngeal and head corpses in Table 1c, only young larvae with four cells in the gonad, that is, between hatching and the middle of the first larval stage, were scored (Kimble and Hirsh, Dev. Biol. 70:396-417 (1979)). For ventral cord corpses (descendants from the blast cells P9-P12) and for tail corpses, third larval stage animals were scored. Extra cells are the number of extra cells among the descendants of P9, P10, and P11. The divisions of these blast cells generate four programmed cell deaths in the wild-type (Figure 6a).

In Table 1d, HSN missing (%) is the percent of missing or grossly displaced HSN neurons. Only first or second larval stage animals were scored. There are two HSNs per animal, one on each side (White et al., Phil. Trans. R. Soc. B311:1-340 (1986)). To score egg laying, staged worms were grown at 20°C. Animals were observed using a dissecting microscope on the second day of adulthood, and those bloated with late-stage eggs were considered egg-laying-defective (Trent and Horvitz, Genetics 104:619-647 (1983)). The alleles used were: ced-1(e1735), ced-3(n717), ced-4(n1162), ced-5(n1812), ced-9(n1950), dpy-17(e164), dpy-18(e364), eql-1(n478sd, ts), lon-1(e1820), unc-36(e251). eT1(e873), a translocation chromosome with a breakpoint that disrupts unc-36 gene function, prevents crossing over on the right arm of chromosome III (Rosenbluth and Baillie,

Genetics 99:415-428 (1981)). nDf40 is a new deficiency which was isolated as a cis-acting suppressor of n1950.

Animals were anaesthetized with 30 mM NaN<sub>3</sub> (Avery and Horvitz, Cell 51:1071-1078 (1987)) and observed  
5 using Nomarski optics microscopy (Sulston and Horvitz, Devl. Biol. 56:110-156 (1987)). Average numbers are shown with, if appropriate, their 95% confidence limits, as determined by the t-test using the StatViewII program (Abacus Concepts, Berkeley, California).

10

## EXAMPLE 2

### Isolation of ced-9(lf) Mutations

Because the ced-9(n1950) mutation causes a gain of gene function (see above), mutations that reduce or eliminate ced-9 activity (ced-9 loss-of-function (lf)  
15 mutations) were isolated by screening for cis-dominant suppressors of ced-9(n1950). Second mutations in ced-9 were expected to be isolated which could suppress the dominant effects of n1950 by inactivating ced-9 (Figure 7). After screening 9,000 haploid genomes, three  
20 candidate suppressor mutations were isolated which were tightly linked to ced-9(n1950). One of these mutations, nDf40, behaved genetically as a large deletion (see below), indicating that the screening procedure should allow the isolation of mutations that completely  
25 inactivate ced-9. The other two mutations, n2077 and n2161, seem likely to be ced-9 loss-of-function alleles: these two mutations failed to complement each other while complementing recessive mutations in all known genes in this region. The n2077 and n2161 mutations  
30 mapped within 0.1 map units of the original n1950 mutation, and were obtained at a frequency of about  $3 \times 10^{-4}$  per haploid genome, which is comparable to that for loss-of-function mutations in other C. elegans genes

-33-

(Brenner, Genetics 77:71-94 (1974); Meneely and Herman, Genetics 92:99-115 (1979); Greenwald and Horvitz, Genetics 96:147-164 (1980)).

It was then determined that another mutation,  
5 n1653ts, which was previously isolated in an unrelated  
screen for mutants with displaced or missing HSN neurons  
(Desai et al., Nature 336:638-646 (1988)), was also a  
ced-9(lf) allele. n1653 was shown to be allelic to  
n2077 and n2161 based on its position on the genetic  
10 map, the similarity of its phenotype at restrictive  
temperature to the phenotypes of n2077 and n2161 mu-  
tants, and its failure to complement n2077 and n2161.  
Programmed cell deaths occurred normally in n1950  
n2077/++ and n1950 n2161/++ animals, but were blocked in  
15 n1653/n1950 trans-heterozygotes. The results of this  
cis-trans test demonstrate that the allelic mutations  
n2077, n2161 and n1653 are in the ced-9 gene, which is  
defined by the mutation n1950, rather than in a closely  
linked gene.

## 20 Methods

The screen for mutations that resulted in a loss of  
ced-9 function (see Figure 7) was performed as follows.  
The semidominant mutation eql-1(n487sd) causes the two  
HSN neurons to die by programmed cell death, so that the  
25 animal bloats with eggs (Ellis and Horvitz, Cell 44:817-  
829 (1986); Trent et al., Genetics 104:619-647 (1983)).  
Because ced-9(n1950) dominantly suppresses eql-1(n487)  
by preventing the deaths of the HSN neurons, only  
animals that do not have ced-9(n1950) function will  
30 bloat with eggs as a result of the eql-1 mutation. Such  
egg-laying defective animals were screened by mating  
eql-1(n487) V males either with unc-69(e587) ced-  
9(n1950) III; unc-10(e102) xol-1(v9) dpy-6(e14) X  
hermaphrodites or with unc-69(e587) ced-9(n1950) dpy-

18(e364) III; lon2(e678) xol-1(y70) X hermaphrodites. Egg laying-defective cross-progeny were picked and their progeny examined for any unusual phenotype. The xol-1 mutation on the X chromosome causes male lethality

5 (Miller *et al.*, *Cell* 55:167-183 (1988) and so prevents mating among F<sub>1</sub> animals, which would complicate genetic analysis of new mutations. The unc-69, dpv-18, lon-2, unc-10, and dpv-6 mutations were used as closely linked genetic markers to identify the chromosomes carrying the

10 ced-9 and xol-1 mutations. General genetic methods and techniques for mutagenesis with ethyl methanesulphonate are described in Brenner, *Genetics* 77:71-94 (1974). Two-factor mapping experiments showed the new mutations n2077 and n2161 to be tightly linked to ced-9(n1950).

15 This screen also generated nDf40, a deficiency that fails to complement unc-69, ced-9, unc-49 and several adjoining genes. The loss-of-function mutation ced-9(n1950 n2077) complements the nearby mutations unc-50(e306), ooc-4(e2078) and emb-25(q45ts); ced-9(n1653ts)

20 complements unc-69(e587). The ooc-4 mutation causes a defect in oogenesis, resulting in hermaphrodite sterility. The mutation emb-25(q45ts) is described in Cassada *et al.*, *Dev. Biol.* 84:103-205 (1981). All other mutations are described in Brenner (1974) *supra*.

25

### EXAMPLE 3

#### The ced-9(lf) Alleles Cause Ectopic Cell Deaths

Animals homozygous for ced-9(lf) mutations show several defects (Table 2). Most obviously, homozygous ced-9(lf) mutants derived from ced-9(lf)/+ heterozygous

30 mothers hatch and grow to normal size, but generate very few eggs (partial sterility), all of which eventually die, usually during embryogenesis (maternal effect lethality). Furthermore, such first generation ced-

-35-

9(lf) animals lack many cells normally present in wild-type animals, resulting in a number of additional defects. For example, many ventral cord motor neurons involved in the control of movement are missing, resulting in uncoordinated body movement. The HSN neurons are missing in hermaphrodites, causing an egg-laying defect (Table 2). Similarly, cells are absent from the male tail, resulting in missing or deformed rays (Table 2). Furthermore, several neurons are sometimes missing from the lumbar ganglion, although their absence does not result in an obvious behavioral abnormality.

To determine why cells are missing, ced-9(lf) animals were observed as they developed. The pattern of cell divisions in wild-type C. elegans is highly reproducible among individuals, and deviations from the normal cell lineage can be identified (Sulston and Horvitz, Dev. Biol. 56:110-156 (1977); Sulston et al., Dev. Bio. 100:64-119 (1983); Kimble and Hirsh, Dev. Biol. 70:396-417 (1979); Sulston et al., Devl. Biol. 78:542-576 (1980)). The studies revealed that many cells that normally survive in wild-type animals instead undergo programmed cell death in ced-9(lf) animals.

For example, the divisions of the 12 ventral cord blast cells P1-P12 (collectively called Pn) were monitored. During the first larval (L1) stage, each P cell divides to generate an anterior daughter (Pn.a) that is a neuroblast and a posterior daughter (Pn.p) that is a hypodermal blast cell. The Pn.a cells then follow identical patterns of divisions to generate motor neurons involved in locomotion (Sulston and Horvitz (1977) supra; White et al., Phil. Trans. R. Soc. 311:1-340 (1986); Chalfie and White, in: The Nematode Caenorhabditis elegans (eds. Wood et al.), pp.337-391 Cold Spring Harbor Laboratory Press, New York, 1988).

Numerous ectopic cell deaths were observed in all Pn.a lineages of ced-9(lf) animals, and frequently all descendants of the Pn.a neuroblasts died.

Ectopic programmed cell death was also observed in  
5 the ray lineages. Rays are simple sensory structures located in the male tail, which is used for copulation. Each of the 18 rays arises from a single ray precursor cell (Sulston and Horvitz (1977) supra). Many ectopic cell deaths occurred in the ray lineages of ced-9(lf)  
10 males. These ectopic deaths often eliminated the ray structure cell, which is required for ray formation (Sulston and Horvitz (1977) supra). Thus, these deaths account for the absence of rays in ced-9(lf) males.

To determine the cause of the maternal-effect  
15 lethality of ced-9(lf) mutations, the embryonic cell lineages of the progeny of ced-9(lf) animals were studied. Embryos generated by mothers homozygous for the weak allele ced-9(n1950 n2161) usually arrested during the early stages of embryo elongation (about 450  
20 minutes after fertilization; Sulston et al. (1983) supra), although there was some variation from animal to animal. These embryos developed normally to about the 200-cell stage, at which point extensive ectopic cell deaths began to appear. These ectopic cell deaths were  
25 morphologically similar to the cell deaths that occur during normal C. elegans development and that also first appear at this stage (Sulston et al. (1983) supra). The cell lineage of a single ced-9(n1950 n2161) embryo was analyzed using a 'four-dimensional'-microscope (which  
30 allows time-lapse recording of multiple focal planes of a specimen. 49 of the cells that died (more than 100 cells died eventually) were identified. Of these 49 dying cells, 45 normally survive in the wild-type. These 45 ectopic deaths prevented the generation of 78  
35 cells, 50 of which would have been neurons or glial

-37-

cells. Mothers of hypodermal cells and of muscle cells, also died. No obvious pattern to the ectopic cell deaths could be discerned. Many of these deaths involved cells that in the wild-type do not generate any descendants that die. Therefore, these deaths were not simply consequences of premature activation of the pathway for programmed cell death.

Embryos from mothers homozygous for the strong allele ced-9(n1950 n2077) were also studied. The n2077 mutation probably results in a complete or nearly complete inactivation of ced-9, because n2077 behaves like the ced-9 deletion nDf40 when placed in trans to each of the ced-9 alleles (Table 2). Surprisingly, the defects and terminal phenotype associated with this allele were quite different from those of n1950 n2161 embryos. The  $F_2$  n1950 n2077 embryos arrested much earlier in development, with different individuals having from a few dozen to a few hundred cells at most. The embryos invariably looked sick, with swollen cells and abnormal granules in the cytoplasm. Furthermore, cell divisions were slow and asynchronous. In those rare animals that developed sufficiently far before arresting, cell corpses started to appear at about the same stage as in n1950 n2161 embryos. The lineage of a single ced-9(n1950 n2077) embryo was followed with the four-dimensional-microscope. This embryo arrested with 57 cells. Nothing resembling a programmed cell death was observed. However, blocks in mitosis and cytokinesis were apparent, with incomplete cytokineses resulting in the formation of several binucleate cells. It is not known whether these defects in cell divisions and the general sickness are effects of a lack of ced-9 function in the embryo or are secondary consequences of abnormalities in the maternal germline. Because all of these defects are completely suppressed by mutations in

ced-3 or ced-4 (see below), it seems likely that they are a consequence of the ced-9(n1950 n2077) allele rather than of another mutation carried in this strain. It is likely that these defects are caused by the  
5 ectopic activation of the pathway for programmed cell death in the maternal germline. Alternatively, the three genes ced-9, ced-3 and ced-4 might act not only in programmed cell death but also in an aspect of early C. elegans development that is unrelated to programmed cell  
10 death.

#### Methods

The loss of ced-9 function results in ectopic cell deaths. Cell lineages of the ventral cord blast cells P1-P12 in the wild-type (Sulston and Horvitz, Dev. Biol.  
15 56:110-156 (1977)) and cell lineages of P1-P12 in a ced-9(n1950 n2077) hermaphrodite progeny of a gC1/unc-69 ced-9(n1950 n2077) heterozygous mother were studied. The exact pattern of cell deaths varied slightly among the three mutant animals studied. Ray lineages in the  
20 wild-type (Sulston and Horvitz (1977) supra; Sulston et al., Dev. Biol. 78:542-576 (1980)) were examined, as well as cell lineages of the left and right R cells, R4L-R9L and R4R-R9R, respectively, in a single ced-9(n1950 n2077) male progeny of a gC1/unc-69 ced-9(n1950  
25 n2077) heterozygous mother crossed with males of identical genotype. The left and right R1-R3 cell lineages were not followed in this particular animal. As in the ventral cord lineages, the exact pattern of ectopic cell deaths varied among the three mutant animals studied.  
30 Also examined were male tails. Unc-69 male tails had nine rays on each side. The male tail of a particular unc-69 ced-9(n1950 n2077) animal has only three rays on the left side and five on the right side. A ced-4 unc-69 ced-9(n1950 n2077) male tail showed 18 rays. In



-39-

wild-type embryos, 350 minutes after fertilization, only a few cell corpses could be seen in ventral view. In ced-9(n1950 n2161) embryos, generated by a ced-9(n1950 n2161) mother, many corpses could be seen 350 minutes after fertilization.

Cell lineages were followed using Nomarski optics microscopy (Sulston and Horvitz (1977) supra). Four-dimensional-microscopy of embryos was done as follows: freshly fertilized embryos were mounted for observation on 5% agar pads in a drop of M9 or egg salts (Sulston et al., Dev. Biol. 100:64-119 (1983)). Pictures of the developing embryos were taken in 18 focal planes (roughly 1  $\mu$ m apart) at 30 second intervals using an apparatus developed by J. G. White and stored on a 12-inch optical video disk for subsequent analysis.

The data for Table 2 were obtained as follows. For Table 2a, the numbers of eggs laid by first generation ced-9(lf) hermaphrodites and the stages of development at which the progeny of these hermaphrodites arrested were examined. First generation hermaphrodites were transferred to fresh plates every 12 hours, and the number of eggs they laid were counted. Note that the absence of HSNs retards but does not prevent egg-laying (Trent et al., Genetics 104:619-647 (1983)), so that the sterility observed in ced-9(lf) animals as reflected by the number of eggs laid per animal cannot be only an effect of the defect in egg release. For example, egl-1(n487sd, ts) animals, which are egg laying-defective because they lack HSNs, nonetheless lay an average of 204 eggs (Desai and Horvitz, Genetics 121:703-721 (1989)). Egg-laying defective ced-9(lf) animals do, however, fertilize a few eggs that are never laid. For example, although wild-type hermaphrodites lay all fertilized eggs within 4 days of reaching adulthood, by the seventh day ced-9(n1950 n2077) hermaphrodites still

had  $1.7 \pm 1.3$  eggs (number of broods = 12) remaining in utero, and egg-laying defective ced-9(n1950 n2161) hermaphrodites had  $30 \pm 23$  eggs (number of broods = 6). The number of eggs laid by ced-9(lf) animals therefore usually slightly underestimates actual brood size. The percent of eggs laid that hatched within 48 hours of removal of the mother were examined; wild-type eggs hatch about 14 hours after fertilization (Sulston and Horvitz, Devl. Biol. 100:64-119 (1983)). The percent of hatched progeny that failed to develop past the first (L1) larval stage within 6 days of hatching were also examined; wild-type larvae remain in the L1 stage for about 12 hours (Sulston and Horvitz, Devl. Biol. 100:64-119 (1983)).

For Table 2b, the percent of animals defective in egg-laying was scored as in Table 1. Note however that for some genotypes (marked †) a significant fraction of the animals could not be scored accurately for egg-laying capability because of the small number of eggs they generated. Egg-laying defective ced-9(lf) animals do lay eggs in the presence of serotonin (assayed as in Trent et al., Genetics 104:619-647 (1983)), suggesting that the serotonergic HSN neurons are defective or absent.

For Table 2c, young adult males were anaesthetized in 30 mM  $\text{NaN}_3$ , placed on their backs and observed using Nomarski optics. All strains were homozygous (nDf40 strains were hemizygous) for the closely linked mutation unc-69(e587), which facilitates identification of the chromosome carrying the ced-9 mutation. All ced-9(lf) were maintained as heterozygous stocks balanced by the chromosome III balancer gC1. The nDf40 chromosome was marked with dpv-18(e364). nDf40 fails to complement both ced-9 and unc-69. For the n2161/n2077 and n1653/n2077 trans-heterozygotes, the maternally-

-41-

inherited n2077 chromosome was marked with the dpy-18(e364) mutation to distinguish self- from cross-progeny. The HSN counts for the n2161/n2077 and n1653/n2077 genotypes were not determined because of the difficulty of scoring the Dpy phenotype in early larvae. The ced-9(+)/Df larvae that arrested as L1s did so as a consequence of the unc-69(e587) mutation, which decreases brood size and results in an incompletely penetrant L1-arrest phenotype when heterozygous with nDf40; by contrast, nDf40/unc-69(+) animals do not arrest development as L1 larvae.

## EXAMPLE 4

The ced-9 Gene Antagonizes ced-3 and ced-4

If the defects associated with a loss of ced-9 function are caused entirely by the aberrant activation of the programmed cell death pathway, then mutations that prevent the process of programmed cell death might be able to suppress these defects. To test this hypothesis, double mutants were constructed using ced-9(n1950 n2077) and mutations in either ced-3 or ced-4, two genes required for programmed cell death (Ellis and Horvitz, Cell 44:817-829 (1986)). Mutations in ced-3 or ced-4 completely suppressed all defects observed in ced-9(n1950 n2077) animals (Table 3, Figure 8g). Similar results were obtained for ced-9(n1950 n2161) and ced-9(n1653ts). These observations suggest that the defects seen in ced-9(lf) animals are indeed caused by the activation of the programmed cell death pathway. Furthermore, if these three genes are part of a regulatory pathway, these results indicate that ced-9 acts before ced-3 and ced-4, because the activities of these genes are required for ced-9(lf) mutations to have their effects.

### Methods

The data for Table 3 were obtained as follows. The numbers of eggs laid were determined as described for Table 2. Viable progeny are the number of progeny that  
5 grew to the fourth larval (L4) stage within 10 days of hatching (this value includes a few animals that developed from eggs that hatched internally); wild-type larvae reach the L4 stage within 2 days. (Sulston and Horvitz, Devl. Biol. 56:110-156 (1977)). Confidence  
10 limits (95%) were determined as in Table 1. Note that ced-3 and ced-4 are able to suppress the ced-9(lf) zygotic defects in a semidominant fashion: animals homozygous for ced-9(n1950 n2077) but carrying only one wild-type copy of either the ced-3 or ced-4 genes showed  
15 milder zygotic defects than did animals with two wild-type copies of both genes, suggesting that lowering ced-3 or ced-4 activity can compensate for lower levels of the Ced-9 protein in first generation ced-9(lf) animals. However, one copy of ced-3 or ced-4 is not sufficient to  
20 suppress the maternal-effect lethality: all the viable progeny generated from ced-9(lf); ced-3/+ mothers were homozygous for the ced-3 mutation. Double mutants were also constructed between ced-9(n1950 n2077) and ced-3(n718), ced-3(n1040), ced-3(n1128), ced-3(n1949), ced-4(n1894), or ced-4(n1920), and between ced-9(n1950 n2161) and ced-3(n717), ced-4(n1162), ced-4(n1894), or ced-4(n1920). All of these double mutants were both  
25 viable and fertile, showing that the suppression of the ced-9(lf) defects by ced-3 and ced-4 is not allele-specific.  
30

-43-

## EXAMPLE 5

Cloning and Sequencing the ced-9 Gene and cDNACloning of ced-9

ced-9 was genetically mapped to the right arm of chromosome III, approximately 0.05 map units to the right of unc-69. This position placed ced-9 between the two cloned genes lin-12 and tra-1. The whole interval between these two genes, corresponding to approximately 2 Mb, had previously been cloned as part of the C. elegans physical mapping effort (Coulson *et al.*, *Proc. Natl. Acad. Sci. USA* 85:4397-4401 (1986); Coulson *et al.*, *Nature* 335:184-186 (1988); Coulson *et al.*, *BioEssays* 13:413-417 (1991)). To narrow down the region containing ced-9, ced-9 was mapped with respect to a series of restriction fragment length polymorphisms (RFLPs) between the common laboratory strain Bristol N2, and RC301, a strain isolated from the wild near Freiburg, Germany.

Mapping of ced-9 with respect to these RFLPs (Figure 9b) localized the gene to a roughly 60 kb interval located between nP55 and nP56. Cosmids from this region were then tested for their ability to rescue the ced-9(lf)-associated defects. Three overlapping cosmids were found to be able to rescue ced-9 (Figure 9c). Various fragments from the region common to all three cosmids were subcloned into plasmids and tested for rescue activity. A 4.7 kb SacI-XhoI fragment was identified in this manner that efficiently rescued both the sterility and the lethality of ced-9(n1950 n2077) mutants. Further deletions into this fragment from either the right or the left abolished or greatly diminished the rescuing the activity of the fragment (Figure 10b).

### ced-9 cDNAs

A 4.2 kb ClaI-XhoI fragment was used to probe a C. elegans cDNA library. Three distinct classes of cDNAs were isolated, corresponding to the 0.75 kb, 1.3 kb, and 2.2 kb transcripts identified on Northern blots. One cDNA of each class was sequenced. The deduced intron/exon structure of the three classes of cDNAs is shown in Figure 10c. Both the 0.75 kb and 2.3 kb cDNAs sequenced had SL1 trans-spliced leaders at their 5' ends, suggesting that full-length cDNAs were isolated. The three cDNA classes are related to each other in an unusual way: the 0.75 and 2.2 kb transcripts share the same 5' end and open reading frame and are predicted to encode identical proteins. The 1.3 kb and 2.2 kb transcripts are predicted to share the same polyadenylation site.

### METHODS:

#### Mutations and Strains

All mutations were generated in a Bristol N2 background, which was used as the standard wild-type strain, except where noted. The following mutations were used:

LGIII:     unc-69(e587), ced-9(n1950dm), ced-9(n1950 n2077), ced-9(n1950 n2161), unc-49(e382).

Mutations other than ced-9 are described in (Brenner, Genetics 77:71-94 (1974)). The ced-9(n1950 n2077) and ced-9(n1950 n2161) mutations were maintained as balanced strains over the LGIII balancer gC1, which carries the mutations dpy-19(e1259ts.mat) glp-1(q339). Strains were maintained as described (Brenner, 1974 supra). All strains were grown at 20°C.

-45-

### RFLP Mapping

Various cosmids from the lin-12 to tra-1 interval were tested for their ability to detect RFLPs between the common laboratory strain N2 and various strains  
5 isolated from the wild and known to contain a large number of transposon-induced RFLPs. The position of ced-9 was then determined relative to these markers as described (Ruvkun *et al.*, Genetics 121:501-516 (1989)). Briefly, N2/RC301 recombinants in the ced-9 region were  
10 obtained by mating RC301 males with unc-69(e587) ced-9(n1950) unc-49(e382) hermaphrodites to generate unc-69 ced-9 unc-49 [N2]/+++[RC301] heterozygotes. From these heterozygotes, Unc-49 non-Ced-9 non-Unc-69 and Unc-49 Ced-9 non-Unc-69 recombinants were cloned, homozygosed  
15 for the recombinant chromosome, and the genotype of the various RFLP loci analyzed by genomic Southern blot analyses.

### Germline Transformation of ced-9 Mutants

DNAs to be tested for ced-9 rescue activity were  
20 microinjected into the mitotic germline of hermaphrodites according to the method developed by Mello and colleagues (Mello *et al.*, EMBO J. 10:3959-3970 (1991)). The relevant DNA was injected at a concentration of 5-25 µg/ml. pRF4, a plasmid containing  
25 a dominant rol-6 mutation, was co-injected as a dominant marker to identify transgenic animals. Since ced-9(lf) mutants are almost sterile and produce only dead progeny, heterozygotes of genotype qC1 dpy-19(e1259)/unc-69(e587) ced-9(n1950) (2077) were injected,  
30 where the unc-69 was used as a linked marker to identify the ced-9 chromosome. Non-Unc non-Dpy Roller F1s were picked to establish stably transmitting roller lines. From these, Roller Unc-69 animals were picked and tested for rescue of the ced-9(lf)-associated sterility and

maternal effect lethality. A clone was considered to rescue if a stable homozygous line of genotype unc-69 ced-9(lf) III; array could be established.

### Molecular Biology

5        Standard molecular biology protocols (see (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989) was followed except where noted. All plasmid subcloning was done into pBluescript vectors (Stratagene).

10        The 4.7 kb genomic SacI-XhoI fragment with rescuing ability was subcloned into pBluescript II (Stratagene) and both strands were sequenced using the ExoIII-S1 nested deletion method and T7 polymerase (Sequenase, USB) following the protocol suggested by the  
15 manufacturer.

      A 4.2 kb ClaI-XhoI rescuing genomic fragment was used to probe a  $\lambda$  cDNA library. From approximately 300,000 plaques, 11 cDNAs were isolated. The sequences present at the ends of the inserts were determined for  
20 all 11 cDNAs. 8 cDNAs corresponded to the 0.75 kb cytochrome b560-like transcript, two (one incomplete) were from the 1.3 kb ced-9 transcript, and one corresponded to the 2.2 kb "fusion" transcript. One cDNA from each class was then completely sequenced (one  
25 strand only).



-47-

TABLE 1  
The Gain-of-Function Allele *ced-9(n1950)* Prevents Programmed Cell Deaths

(a) <i>ced-9(n1950)</i> prevents programmed cell deaths			
Maternal genotype	Zygotic genotype	Extra cells in anterior pharynx	No. of Animals
<i>ced-3/ced-3</i>	<i>ced-3/ced-3</i>	12.5±0.7	30
<i>ced-4/ced-4</i>	<i>ced-4/ced-4</i>	13.9±0.5	40
<i>ced-9(n1950)/ced-9(n1950)</i>	<i>ced-9(n1950)/ced-9(n1950)</i>	13.3±0.6	45
(b) <i>ced-9(n1950)</i> is a dominant gain-of-function mutation and shows a maternal effect			
Maternal genotype	Zygotic genotype	Extra cells in anterior pharynx	No. of Animals
+/+	+/+	0.03±0.05	60
	<i>Df/+</i>	0.00	50
	<i>ced-9(n1950)/+</i>	5.3±0.8	25
<i>ced-9(n1950)/+</i>	+/+	0.2±0.22	25
	<i>ced-9(n1950)/+</i>	11.4±0.8	30
	<i>ced-9(n1950)/ced-9(n1950)</i>	13.7±0.5	30
<i>ced-9(n1950)/ced-9(n1950)</i>	<i>ced-9(n1950)/+</i>	11.8±0.6	30
	<i>ced-9(n1950)/ced-9(n1950)</i>	13.3±0.6	45

TABLE 1 (continued)

(c) *ced-9(n1950)* suppresses the accumulation of cell corpses

Genotype	Corpses		Corpses		n
	Pharynx	n	Head	n	
Wild-type (N2)	0	50	0.0±0.1	50	
<i>ced-1</i>	0.8±0.2	100*	28	10†	
<i>ced-1; ced-3</i>	0.02±0.04	50	0.3±0.1	50	
<i>ced-1; ced-4</i>	0.02±0.04	50	0.7±0.2	50	
<i>ced-1; ced-9(n1950)</i>	0	30	0.5±0.3	30	
<i>ced-5</i>	3.6±0.6	25	16±5	10	
<i>ced-5; ced-3</i>	0.1±0.1	40	0.5±0.2	40	
<i>ced-4; ced-5</i>	0.2±0.2	40	1.0±0.3	40	
<i>ced-9(n1950); ced-5</i>	0.1±0.1	100	0.8±0.4	25	

Corpses			Extra Cells	
P9-P11	P12	Tail	P9-P11	n
0	0	0	0	30
3.5±0.3	1.7±0.3	1.7±0.3	0.4±0.3	30
0.03±0.07	0	0.3±0.2	3.9±0.1	30
0.03±0.07	0	0.3±0.2	4.0±0.1	30
0	0	0.3±0.2	4.0±0.1	30
3.0±0.5	2.2±0.3	4.6±0.8	0.2±0.2	21
0.05±0.10	0	0.4±0.4	3.9±0.1	20
0.05±0.10	0	1.6±0.5	3.9±0.2	20
0	0	1.1±0.3	3.8±0.1	30

TABLE 1 (continued)

(d) *ced-9(n1950)* prevents the deaths of the HSN neurons in *egl-1* mutants

Genotype	HSNs missing (%)	No. of sides	Egg-laying defective (%)	n
Wild-type (N2)	1	250	0.4	704
<i>egl-1</i>	99	200	99	447
<i>ced-3; egl-1</i>	0	160	0.2	599
<i>ced-4; egl-1</i>	0	100	0	417
<i>ced-9(n1950); egl-1</i>	0	200	0	417

-49-

a. The genotypes of animals studied were as shown.

b. The complete genotypes are given in Example 1.

c. Extra cells, number of extra cells among the descendants of P9, P10, and P11.  
n, number of animals scored. \*Data from Ellis *et al.*, Genetics 129:79-94 (1991).  
†Data from Ellis and Horvitz, Cell 44:817-829 (1986).d. HSN missing (%), percent of missing or grossly displaced HSN neurons. No. of sides,  
number of sides scored. n, number of animals scored.

Average numbers are shown with, if appropriate, their 95% confidence limits.

TABLE 2  
Phenotypes of *ced-9(lf)* Mutants

Genotype	<i>ced-9(+)</i>	<i>ced-9(+)</i> Df		<i>n1950 n2161</i>					<i>n1950 n2161</i> Df		<i>n1950 n2161</i> <i>n1950 n2077</i>		
		20°C	20°C	20°C	15°C	20°C	23°C	25°C	20°C	20°C	20°C	20°C	
(a) Sterility and maternal-effect lethality													
Eggs laid per animal	209±33	202±60	117±36	97±31	45±22	6.3±4.5	23±14	40±9					
Hatching (%)	99±1	75±2	12±3	2.4±0.7	0.4±0.4	0	0	0					
L1 arrest (%)	0	13±3	100	100	100	NA	NA	NA					
	n=14	n=9	n=23	n=42	n=36	n=60	n=50	n=49					
(b) Egg-laying defect													
Egg-laying defective (%)	0	0	64±16	76±13	94±7	98±3	96±4	96±6					
	n=100	n=35	n=23	n=42	n=36	n=60	n=50	n=40					
HSNs missing (%)	0	0	77	87	94	95	95	ND					
	n=100	n=48	n=118	n=138	n=100	n=130	n=60						
(c) Absence of rays in male tails													
Rays per side	8.9±0.1	8.6±0.2	8.0±0.3	6.6±0.3	5.9±0.3	5.4±0.4	6.0±0.3	5.9±0.3					
	n=68	n=34	n=40	n=40	n=58	n=34	n=62	n=38					

-51-

TABLE 2 (continued)  
Phenotypes of *ced-9(lf)* Mutants

Genotype*	<i>n1653ts</i> 25°C	$\frac{n1653ts}{Df}$ 25°C	$\frac{n1653ts}{n1950\ n2077}$ 25°C	<i>n1950 n2077</i> 20°C	$\frac{n1950\ n2077}{Df}$ 20°C
(a) Sterility and maternal-effect lethality					
Eggs laid per animal	2.7±1.1	0	0.3±0.4	1.6±1.4	0.8±1.6
Hatching (%)	38±24	NA	0.7±1.4	0	0
L1 arrest (%)	40±36	NA	100	NA	NA
	n=26	n=15	n=30	n=20	n=24
(b) Egg-laying defect					
Egg-laying defective (%)	NA†	NA†	NA†	NA†	NA†
HSNs missing (%)	ND	ND	ND	99	100
	n=38	n=44	n=52	n=220	n=42
(c) Absence of rays in male tails					
Rays per side	8.6±0.2	7.6±0.3	8.1±0.3	4.6±0.3	4.9±0.6
	n=38	n=44	n=52	n=81	n=26

SUBSTITUTE SHEET

TABLE 2 (continued)

- a. Hatching (%), percent of eggs laid that hatched within 48 hours of removal of the mother.  
L1 arrest (%), percent of hatched progeny that failed to develop past the first (L1) larval stage within 6 days of hatching. n, number of broods analysed.
- b. For some genotypes (marked †) a significant fraction of the animals could not be scored accurately for egg-laying capability. n, number of animals scored. HSN missing (%), percent of missing or grossly displaced HSN neurons. n, number of sides scored.
- c. Rays per side, number of rays present per side in the male tail. n, number of sides scored.  
Confidence limits. (95%) were determined as in Table 1. NA, not applicable. \*All strains were homozygous (*nDf40* strains were hemizygous) for the closely linked mutation *unc-69(3587)*.

TABLE 3  
Mutations in *ced-3* and *ced-4* Suppress the Defects Resulting From the Loss of *ced-9* Function

Genotype*	Sterility and Maternal-effect lethality			Egg-laying defect			Male tail	
	Eggs laid per animal	Viable progeny	n	Egg-laying defective (%)	n	Rays per side	n	
<i>ced-9(+)</i>	207±36	207±33	14	0	100	8.9±0.1	68	
<i>ced-9(n1950 n2077)</i>	1.6±1.4	0	20	NA†	100	4.6±0.3	81	
<i>ced-4 ced-9(n1950 n2077)</i>	200±19	160±20	12	1	84	9.0	42	
<i>ced-4</i>	182±17	148±17	12	0	100	9.0±0.1	30	-53-
+ <i>ced-9(n1950n 2077)</i>								
<i>ced-4 ced-9(n1950 n2077)</i>	56±20	3.9±2.0	7	62	71	7.4±0.3	50	
<i>ced-9(n1950 n2077); ced-3</i>	154±29	94±22	13	0	100	9.0	28	
<i>ced-3</i>	178±51	146±46	6	0	66	8.9±0.1	50	
<i>ced-9(n1950 n2077); ced-3/+</i>	132±62	17±6	10	18	40	8.7±0.1	50	

Viable progeny, number of progeny that grew to the fourth larval (L4) stage within 10 days of hatching (this value includes a few animals that developed from eggs that hatched internally);

n, number of broods analysed.

Confidence limits (95%) were determined as in Table 1.

\*All strains are homozygous for the closely linked mutation *unc-69(e587)*.

†Many animals could not be accurately scored for egg-laying.

-54-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT

(A) NAME: MASSACHUSETTS INSTITUTE OF TECHNOLOGY  
 (B) STREET: 77 Massachusetts Avenue  
 (C) CITY: Cambridge  
 (D) STATE OR PROVINCE: Massachusetts  
 (E) COUNTRY: U.S.A.  
 (F) POSTAL CODE: 02139

(ii) TITLE OF INVENTION: A Gene Which Prevents Programmed Cell Death

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: diskette

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6560 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCGATAGTC GTCACCAAAT GGATTTTCCG ATTTCTCACT AGTCCATGGC TCACAATTTA	60
CAAAATCTCG AGAAAAGAAA GGATGCAAGG AGTATGAAGA GGTTCGGAAT CTAAATATTT	120
TAATTTAAAA AAATCAATTT CGAATTGAAA TTCAACTCCT ACTCGTTTTG AAAATGCCAA	180
TCCTTTAAGT AACTTCTGG ATCGCCCAT TCTTCCAGAA ATTCCTTCAA AGTAGTGGTT	240
TTGTACTGAT TTCCTCCGCA AAGAATAGGA ACTTTCGAAT CTCCTGGAGC GAAACGGGAT	300
TTTSATAACA AAAAATATC CAGACAAACC ATAGGACTTT TTCAAATATT CCTTATTTGG	360
CTGTCCATTT GGAAGCACCC AATCTTTAAC GCTGTCCAGC CAGAAGTGCT CCACTCGCCA	420
AGGATAAAAG GCTCATTTTT GAAGCCGAAT TTTACTAAAA TCTCTAGCCA TGGAGTCGAT	480
GGATCAGAAA TTCGAGGAAT TTTAGATTTC ATCTTGAAAT TTGCAATGGA AAAAATAATT	540
ATTCAAAGAA AATCACAGAA AATGCAACAA AAAAAACAAA AAAAGAACAA AAAACAAGTC	600
GAAAAGTGCG CCCGGGTCGT TTGCTGACGC ATCTCTTCAA ACGAGACGCG CTGCTGGCGC	660
ACTTCTCGTG CCCTGTGCGT GCATTTCCGC AACAAAATTC AACACTTGTT TTGAAACGCA	720
CCGCCCTGTT TCTTTTTTCA ATTTTGATAA GAAATCAGC ATTGTTTCAG GATGATTAAAC	780
ATTCCAAC TGCGATTCTGT CCGCTTGGGC GCCAGATCGT CGATTTCCTG CTCCTTTGGA	840
ACATCGATCG TCACCAAGGT GGGGATTTTT TGAATTTTTT CGTGAAAATT GTTGATTTTT	900



-55-

TGTGTACGCA	TGAAGGAGAA	ATGTATAACA	GACACATTCT	TTTCAATTAA	TTATTTATAA	960
TATTCACAGT	CCGAGGCCAA	GACGCCAATC	CAGAAGTTTC	GATGGGAATA	CCTGTTGAAG	1020
CAGCGCTCCA	AGAATCGCCC	AATCGCTCCA	CATCTCACCG	TCTACCAGCC	ACAATTGACC	1080
TGGATGCTCT	CCGGATTCCA	TAGAATCAGC	GGTTGTGTAA	TGGCCGGAAC	CCTTCTCGTC	1140
GGAGGAATCG	GATTTCGAGT	TTTGCCGTTT	GATTTCACCG	CTTTTGTGGA	TTTCATCCGT	1200
AGCTGGAAT	TACCATGCGC	GGTGACCGCT	GTCTTCAAGT	ACATCATTGC	TTTCCCCATC	1260
ATTTTCCATA	CTCTTAACGG	AATTCGCTTC	TTAGGATTTC	ATTTGGCTAA	GGGAGTCAAT	1320
AATGTTGGAC	AGGTAGGAGT	TGAATTATT	AATTTAATTG	TTTTAAAATA	AAAATTAATT	1380
TTCAGATCTA	CAAATCGGGA	TATCTCGTAT	CTGGACTTTC	GGCTATTCTT	GCTCTCGCCA	1440
TTGTCTTCAA	CTCTTGCCAG	AACAAGAGCA	ACAAGACTGC	CTAGGCACAG	ATGCTCCGCC	1500
TTCTTTTTTC	TTACTCCGCC	CCAGCCCTCG	ACAATTCTCG	TCAATTTACT	TTTACCGTTG	1560
ATTTCTTCGA	TTTTCTCTCT	TTTCCGTAGA	TTTACCTCTC	CTCTTCGTTT	TTTTTTCTCT	1620
GTCTAGAATG	TATATTATGA	TTATGAAAAC	GAATAAAAAT	TTTAGATGAC	ACGCTGCACG	1680
GCGGACAAT	CGCTGACGAA	TCCGGCGTAT	CGGCGACGAA	CGATGGCGAC	TGGCGAGATG	1740
AAGGAGTTTC	TGGGGATAAA	AGGCACAGAG	CCCACCGATT	TTGGAATCAA	TAGTGATGCT	1800
CAGGACTTGC	CATCACCGAG	TAGGCAGGCT	TCGACGCGAA	GAATGTCCAT	CGGAGAGTCA	1860
ATTGATGGAA	AAATCAATGA	TTGGGAAGAG	CCAAGGCTTG	ATATCGAGGG	ATTTGTGGTA	1920
ATTTTTTAAT	TTTTTTTTGT	AAATAAAATT	TCCTGCTGCT	TCCAGGTCGA	CTATTTACAG	1980
CACCGAATCC	GGCAAAACGG	AATGGAATGG	TTTGAGACAC	CGGGATTGCC	GTGTGGAGTG	2040
CAACCGGAGC	ACGAAATGAT	GCGAGTTATG	GGAACGATAT	TCGAGAAGAA	GCACGCGGAA	2100
AATTTTGAGA	CCTTCTGTGA	GCAGCTGCTC	GCAGTGCCCA	GAATCTCATT	TTCACTGTAT	2160
CAGGATGTGG	TTCGGACGGT	TGGAAATGCA	CAGACAGATC	AATGTCCAAT	GTCTTATGGA	2220
CGTTTGGTAA	GGGAGAAAAT	ACTGAAAAAA	AGTTTGCAAA	AATTCGAAAA	TCGCCAGAA	2280
AGGTGGCAGA	AAAAACATTT	GCAAAAATTG	TTTGTTTTCC	TTCAGGAAAT	CAGCAAACT	2340
TGGTCAAAAA	TAGCCCAATT	ATGTGTCTTT	TTTGAAAGTT	TTCCATTAAA	AAACCACGAA	2400
TTTTGATCCC	GGATTGTAAT	TTTTTTTGTT	GATAAATTAG	CAGAAAACCT	TACGAATTCC	2460
ATTAAAAACG	TTATTTTCTA	TTCGAATATT	TTTAAAGCAT	ATTTTCCTTG	ATTTGTATTT	2520
GCGAAAAAGA	TCTGCTGATT	TATCAAAAAT	CGGTTTTTAA	ATGTAAAATT	TGTGGAAAAT	2580
ACATTAAAAT	TCGATTTTTG	AACCTTTTTT	TTCGAAAAAC	AGGTTTTTCT	GCTGATTTCG	2640
TGAACGAAAA	ACCCCAAAAA	TTCAATTTTC	GAACATTAAA	AACCAGAAAA	ATCGTTTTTT	2700
TAAGCTTAAT	TTTCCGCCAG	AAATGAACGA	ATTAAATTGC	AAATTTCTAA	TTTTCAGATA	2760

-56-

GGTCTAATCT	CGTTCGGCGG	TTTCGTAGCT	GCAAAAATGA	TGGAATCCGT	GGAAGTGCAG	2820
GGACAAGTGC	GAAACCTCTT	CGTTTACACA	TCGCTGTTCA	TCAAAACGCG	GATCCGCAAC	2880
AACTGGAAGG	AACACAATCG	GAGCTGGGTA	AGGAGTATTT	GCATAGACAT	TAGAAGTCAA	2940
TATCCCCCTT	TCCCTAGTAC	CCTTGACTTC	CCGGGGTGTT	GGTAAGCCGA	TAATTACAGG	3000
GTTTCGGTAGC	CTCTTGGGGG	GACAGCTGGA	AACATATTCA	AGTATATTAC	TGTTTATGAT	3060
AATGTTATTG	TTACGGGAAT	ACAAAATTCG	CAGAATGCTA	TTTCACAACA	TATTTGACGC	3120
GCAAAATATC	CAGTAGAGAA	AACTACAGTA	ATTCTTTAAA	TTTTTAAAAT	TTTTACAATT	3180
AAAGAAAATA	ACCACTAATC	AAAAGAAATT	AATTTCAAAA	ATCGAGCCCG	TAAATCGACT	3240
ACAGTAGGCA	TTTAAAGAAT	TACTGTAGTT	TTTCGTACGA	GATATTTCCG	CCTCAAATAT	3300
GTTGTGAAAT	ACGCATTAC	GGATTTTGT	GTTCCCGGA	ATATGCTCTA	AAGCATTATT	3360
TGTGAAAATA	AAAAATCAAG	AAAAAAATTG	CAGGACGACT	TCATGACACT	CGGAAAACAA	3420
ATGAAAGAGG	ACTACGAACG	AGCAGAAGCT	GAAAAAGTGG	GACGCCGGAA	GCAGAACAGA	3480
CGGTGGTCGA	TGATTGGCGC	TGGAGTAACA	GCTGGAGCCA	TTGGAATCGT	TGGAGTCGTC	3540
GTGTGTGGGC	GGATGATGTT	CAGCTTGAAG	TAACGTATTC	AATTTGTGTA	AATAATTAAT	3600
TTATGTACAA	CTCCTTACAT	TTGAATCTCA	TTTTTGCTCA	CTGATTCTCT	CATCCTTTGA	3660
ACTGGAAGAA	GTGGGAAAGC	TAGGCCACAA	ATTACGGCTC	TCTGTGTCGA	TTTACGATTT	3720
TACTGCAATT	TTTTCCGATT	GCCTTTTTTT	TTGGCCAAAC	CCTACTTCCG	CGTAATATCA	3780
ACTTTTCCGT	GTTCTGTACA	TTTCGTCAAA	AACCCTGAAA	CCCTAACTTT	TCTCGCCGTG	3840
GCCTAGCCTC	CCGCTTCTCT	TCCACATTTT	CAAAGTACCC	CTGTATCTCA	ATAATTCATC	3900
TTCACTTTAA	CTGTCTCTTT	TCGTGTGGCC	TCTTCCAAC	CCCCCAAAT	TCCTGTACGC	3960
GTACGCGACT	TTGTATTTAT	TTTTTTCAAA	TTGTTTTCTC	TCTACAACAA	CAAAAAAAC	4020
GGTTCTTTTA	TTCAACCCTT	TTTTCGGAAC	GAAACTGCAA	TTTGTATAAT	AGGCGTGCGC	4080
AAGAGAATCC	GGTTTTTATT	TTGCCATCA	CGTCATCCAA	AAAAGTTTAG	TAGGAAAATA	4140
TCATTTTTTA	ATATAATGAT	TCATCTTTCT	CGCCTCTTCT	GTCTCGAGAC	GACGGTCAAT	4200
TCGATGGCCT	TGAATTTTTT	GAAAACAAAA	ATGTTTTTGT	TTAGTGTAAT	CGATCCCCC	4260
GCCTTATCGC	TGTTTCACCA	TCAGATAGGC	TCCGCCATTT	GATTCCCTTG	AATTTGTGCG	4320
GATATATAAA	CAAAAAACGT	TAGTGACCGA	TTCAAAAAAC	AACAATGCGT	GCTTTACTAT	4380
TCACCTCTGT	TGTTCTTTTG	GCTTTGGCTT	TTGTTGAGGC	AAAGAAGCAG	ACTATCACTG	4440
TCAAGGGTAC	AACTATTTGT	AATAAGAAGA	GAATTCAGGM	GRAGGTTACC	TTTGGGAGAA	4500
AGATACTCGT	GAGTTTTTCA	TCTTGTTTAG	CTTGAAACGG	CTTAAAAAGG	ACTAAAAAGG	4560
CCTAAAAATT	GAAGTTTTTC	ACCTGTTTTT	AAAAGAAAGC	CGAATTGCAC	AGCTTTACAC	4620

-57-

GAGATTTCTC	AATAATTTGT	ATTGAAATT	TTCATATTCA	TCCCCAAACG	TTCTTTACAC	4680
GAAATTTTGC	GATTTTGTAG	CTTAAAATAC	GATACCTGGT	CTCGACACGA	AACATTTTGT	4740
TTAAATTCAA	AAAGATGTGC	GCCTTTAAAG	AGTGCTGTAG	TTTGAAACTT	CTGTTGTTGC	4800
GGACTTTTCA	TCGATTTTTC	GTAGCGTTTT	TTTATAAGAA	AAATGTATTT	ATTTATTCAA	4860
AAATTTAATT	TTACCGAATC	GCGAAAAACA	AAATGAAGAA	CACCGATAAA	AATATCGCAG	4920
CAACAATAGT	TTGAAATTAC	AGTACTCTTT	TAAGGNGNNC	ACATTTCCCTA	TATTTACACAC	4980
AAACTTGTCTG	TGTCGNNNCN	GGGTATCGTC	ATTTTGATGC	AGAAATCAAG	AAAATTGCAT	5040
ATATGTTCAA	AAAACCACAA	TTATGGCGAA	TTTCAAGCTT	GAAACGAAAA	TTCAGGAAAT	5100
TCTAAAAATT	AAAAAAAAT	CATTCGAAAT	GTGAAATTTG	ATATTCAACT	TGAAGTCCAT	5160
ATGGCAAATT	TCGTCTATTC	CGNNNTTCGA	NNATTTTGTT	CCACGTGGCC	GCGAAAAGAG	5220
AAAGCACGAN	NACTGATTTC	TGGCAATTTT	TTCCTGTACC	GTGTCAATTA	TTTGAAACTC	5280
TAATAAGCTG	GTATTTTCT	GCTATTGACA	ACTAACTGAA	TCCATAATTT	GCAATTATAA	5340
TATTGACTTT	TGATGTGTGG	CTTAGAAAAA	AAAAACCAAA	AACCTCATCT	AGCTTTAGGC	5400
TGCCAATATA	TTCCTAGGAC	ATATAAAAAA	CCCTTAAAT	TCTCTGCAAC	ACCTACAAGC	5460
TATCAAACGT	ACTATTAGTA	TTCAATTTTC	CAGTCGACCC	CGATGACAAG	CTCGCCTCAA	5520
TGCAATCGAA	CAAAGAAGGA	GAGTTCTCAC	TTACCGGATC	CGACGACGAG	ATCACCTCAA	5580
TCTCTCCATA	CCTCATAATC	ACCCACAAC	GCAACGTGAA	GAAGGCCGGA	TGCAAGCGTG	5640
TTTCAGAGTA	TTTGATTCCA	AAGGAGAAGA	TCGGTGGAA	CTATGATATG	ACATACGTCA	5700
CTCTTGATAT	TCTTTCCGCT	AAAGACAAGG	AGAAGTGCTA	AGAAAATGTT	TTTTTTGTTT	5760
GGTTTGCTTG	TTTGGAAGGG	AAGGACTTTC	TATCTCTTTT	AATTCAACAA	TAACTATTG	5820
GAAAACCGTT	GAAATTTTAA	CCTTGAAC	TAAGAAAAGT	TGCGTGATTA	TGTTGACAAT	5880
TTTGCCAAGT	ATATCTTTGT	GGATATCACA	ATAAACGAAG	TCAAAGCACG	AAATATTACG	5940
GAAACACAAA	ATTAATGAGA	ATGCGCAACA	TATTTGACCG	CAAAATATCT	CGTAGCGAAA	6000
CTACAGTAAT	TCTTCAAAG	ACTACTGTAG	CGCTGTGTCG	ATTTACGAGC	TCGATTTTTG	6060
AAATGAATCA	GACTAGAAGA	AAAGGAGGAA	AATATTGAAC	ATCAATTGAA	CATCAATTCA	6120
AAAAGTCGAA	CCCTTGACTA	CAGTAGTCTT	CTAAAGAATT	ACTGTAGTTT	TCGCTACGAG	6180
ATATTTTGNG	NGTCAAATAT	GTTGNGCAAT	ACGCATCCTC	AGAATTGTGT	GTTCTCGTAA	6240
TGTCTTGAAA	ATTTTCCATT	TCAACATCAA	ATAAGCAAAT	CTAAAAATGT	GGGTTCTGCA	6300
GCGACCACTA	TGACTGTGAT	CGTGGCAAGA	CCCACTCAGA	AAACTACGTG	TTCCTTTAAA	6360
CAAATACATT	TTTAAGTATT	GTAGGTATAA	AAATTGTTGG	CTAGCAGTCT	AGGCTGCCTT	6420
TTTCAGTCGA	CAAACCTCTA	ATTTAATCGG	CGGGTCTTCA	AAAAGTCGTT	TCTTTGAAAA	6480

TATAAAGCTT TATATATTTA TATATTAAAA ATTTTGATTA CATGATATCA AAAGCGACTA 6540  
GTTTGTATAA AAATTATCAA 6560

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1315 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 7..846
- (D) OTHER INFORMATION: /product= "Ced-9"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTGAG ATG ACA CGC TGC ACG GCG GAC AAC TCG CTG ACG AAT CCG GCG	48
Met Thr Arg Cys Thr Ala Asp Asn Ser Leu Thr Asn Pro Ala	
1 5 10	
TAT CGG CGA CGA ACG ATG GCG ACT GGC GAG ATG AAG GAG TTT CTG GGG	96
Tyr Arg Arg Arg Thr Met Ala Thr Gly Glu Met Lys Glu Phe Leu Gly	
15 20 25 30	
ATA AAA GGC ACA GAG CCC ACC GAT TTT GGA ATC AAT AGT GAT GCT CAG	144
Ile Lys Gly Thr Glu Pro Thr Asp Phe Gly Ile Asn Ser Asp Ala Gln	
35 40 45	
GAC TTG CCA TCA CCG AGT AGG CAG GCT TCG ACG CGA AGA ATG TCC ATC	192
Asp Leu Pro Ser Pro Ser Arg Gln Ala Ser Thr Arg Arg Met Ser Ile	
50 55 60	
GGA GAG TCA ATT GAT GGA AAA ATC AAT GAT TGG GAA GAG CCA AGG CTT	240
Gly Glu Ser Ile Asp Gly Lys Ile Asn Asp Trp Glu Glu Pro Arg Leu	
65 70 75	
GAT ATC GAG GGA TTT GTG GTC GAC TAT TTC ACG CAC CGA ATC CGG CAA	288
Asp Ile Glu Gly Phe Val Val Asp Tyr Phe Thr His Arg Ile Arg Gln	
80 85 90	
AAC GGA ATG GAA TGG TTT GGA GCA CCG GGA TTG CCG TGT GGA GTG CAA	336
Asn Gly Met Glu Trp Phe Gly Ala Pro Gly Leu Pro Cys Gly Val Gln	
95 100 105 110	
CCG GAG CAC GAA ATG ATG CGA GTT ATG GGA ACG ATA TTC GAG AAG AAG	384
Pro Glu His Glu Met Met Arg Val Met Gly Thr Ile Phe Glu Lys Lys	
115 120 125	
CAC GCG GAA AAT TTT GAG ACC TTC TGT GAG CAG CTG CTC GCA GTG CCC	432
His Ala Glu Asn Phe Glu Thr Phe Cys Glu Gln Leu Leu Ala Val Pro	
130 135 140	
AGA ATC TCA TTT TCA CTG TAT CAG GAT GTG GTT CGG ACG GTT GGA AAT	480
Arg Ile Ser Phe Ser Leu Tyr Gln Asp Val Val Arg Thr Val Gly Asn	
145 150 155	

-59-

GCA CAG ACA GAT CAA TGT CCA ATG TCT TAT GGA CGT TTG ATA GGT CTA Ala Gln Thr Asp Gln Cys Pro Met Ser Tyr Gly Arg Leu Ile Gly Leu 160 165 170	528
ATC TCG TTC GGC GGT TTC GTA GCT GCA AAA ATG ATG GAA TCC GTG GAA Ile Ser Phe Gly Gly Phe Val Ala Ala Lys Met Met Glu Ser Val Glu 175 180 185 190	576
CTG CAG GGA CAA GTG CGA AAC CTC TTC GTT TAC ACA TCG CTG TTC ATC Leu Gln Gly Gln Val Arg Asn Leu Phe Val Tyr Thr Ser Leu Phe Ile 195 200 205	624
AAA ACG CGG ATC CGC AAC AAC TGG AAG GAA CAC AAT CGG AGC TGG GAC Lys Thr Arg Ile Arg Asn Asn Trp Lys Glu His Asn Arg Ser Trp Asp 210 215 220	672
GAC TTC ATG ACA CTC GGA AAA CAA ATG AAA GAG GAC TAC GAA CGA GCA Asp Phe Met Thr Leu Gly Lys Gln Met Lys Glu Asp Tyr Glu Arg Ala 225 230 235	720
GAA GCT GAA AAA GTG GGA CGC CGG AAG CAG AAC AGA CGG TGG TCG ATG Glu Ala Glu Lys Val Gly Arg Arg Lys Gln Asn Arg Arg Trp Ser Met 240 245 250	768
ATT GGC GCT GGA GTA ACA GCT GGA GCC ATT GGA ATC GTT GGA GTC GTC Ile Gly Ala Gly Val Thr Ala Gly Ala Ile Gly Ile Val Gly Val Val 255 260 265 270	816
GTG TGT GGG CGG ATG ATG TTC AGC TTG AAG TAACGTATTC AATTTGTGTA Val Cys Gly Arg Met Met Phe Ser Leu Lys 275 280	866
AATAATTAAT TTATGTACAA CTCCTTACAT TTGAATCTCA TTTTKGCTCA CTGATTCTCT	926
CATCCTTTGA ACTGGAAGAA GTGGGAAAGC TAGGCCACAA ATTACGGCTC TCTGTGTCTGA	986
TTTACGATTT TACTGCAATT TTTTCCGATT GCCTTTTTTT TTGGCCAAAC CCTACTTCCG	1046
CGTAATATCA ACTTTTCCGT GTTCTGTACA TTTCGTCAAA AACCCTGAAA CCCTAACTTT	1106
TCTCGCCGTG GCCTAGCCTC CCGCTTCTCT TCCACATTTC CAAAGTACCC CTGTATCTCA	1166
ATAATTCATC TTCACTTTAA CTGTCTCTTT TCGTGTGGCC TCTTCCAACCT CCCCCCAAT	1226
TCCTGTACGC GTACGCGACT TTGTATTTAT TTTTTCATAA TTGTTTTCTC TCTACAACAA	1286
CAAAAAAAC GGTTCAAAA AAAAAAAA	1315

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Thr Arg Cys Thr Ala Asp Asn Ser Leu Thr Asn Pro Ala Tyr Arg  
1 5 10 15

Arg Arg Thr Met Ala Thr Gly Glu Met Lys Glu Phe Leu Gly Ile Lys  
20 25 30

Gly Thr Glu Pro Thr Asp Phe Gly Ile Asn Ser Asp Ala Gln Asp Leu  
35 40 45

Pro Ser Pro Ser Arg Gln Ala Ser Thr Arg Arg Met Ser Ile Gly Glu  
50 55 60

Ser Ile Asp Gly Lys Ile Asn Asp Trp Glu Glu Pro Arg Leu Asp Ile  
65 70 75 80

Glu Gly Phe Val Val Asp Tyr Phe Thr His Arg Ile Arg Gln Asn Gly  
85 90 95

Met Glu Trp Phe Gly Ala Pro Gly Leu Pro Cys Gly Val Gln Pro Glu  
100 105 110

His Glu Met Met Arg Val Met Gly Thr Ile Phe Glu Lys Lys His Ala  
115 120 125

Glu Asn Phe Glu Thr Phe Cys Glu Gln Leu Leu Ala Val Pro Arg Ile  
130 135 140

Ser Phe Ser Leu Tyr Gln Asp Val Val Arg Thr Val Gly Asn Ala Gln  
145 150 155 160

Thr Asp Gln Cys Pro Met Ser Tyr Gly Arg Leu Ile Gly Leu Ile Ser  
165 170 175

Phe Gly Gly Phe Val Ala Ala Lys Met Met Glu Ser Val Glu Leu Gln  
180 185 190

Gly Gln Val Arg Asn Leu Phe Val Tyr Thr Ser Leu Phe Ile Lys Thr  
195 200 205

Arg Ile Arg Asn Asn Trp Lys Glu His Asn Arg Ser Trp Asp Asp Phe  
210 215 220

Met Thr Leu Gly Lys Gln Met Lys Glu Asp Tyr Glu Arg Ala Glu Ala  
225 230 235 240

Glu Lys Val Gly Arg Arg Lys Gln Asn Arg Arg Trp Ser Met Ile Gly  
245 250 255

Ala Gly Val Thr Ala Gly Ala Ile Gly Ile Val Gly Val Val Val Cys  
260 265 270

Gly Arg Met Met Phe Ser Leu Lys  
275 280

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5086 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

-61-

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1459..2178

(D) OTHER INFORMATION: /product= "Bcl-2"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGCCCGCCC CTCCGCGCCG CCTGCCCCGC CGCCCGCCGC GCTCCCGCCC GCCGCTCTCC	60
GTGGCCCCGC CGCGCTGCCG CCGCCGCCGC TGCCAGCGAA GGTGCCGGGG CTCCGGGCCC	120
TCCCTGCCGG CGGCCGTCAG CGCTCGGAGC GAACTGCGCG ACGGGAGGTC CGGGAGGCGA	180
CCGTAGTCGC GCCGCCGCGC AGGACCAGGA GGAGGAGAAA GGTGCGCAG CCCGGAGGCG	240
GGGTGCGCCC GTGGGGTGCA GCGGAAGAGG GGGTCCAGGG GGGAGAACTT CGTAGCAGTC	300
ATCCTTTTTA GAAAAGAGG GAAAAATAA AACCTCCCC CACCACCTCC TTCTCCCCAC	360
CCCTCGCCGC ACCACACACA GCGCGGGCTT CTAGCGCTCG GCACCGGCGG GCCAGGCGCG	420
TCCTGCCTTC ATTTATCCAG CAGCTTTTCG GAAAATGCAT TTGCTGTTCG GAGTTTAATC	480
AGAAGACGAT TCCTGCCTCC GTCCCCGGCT CCTTCATCGT CCCATCTCCC CTGTCTCTCT	540
CCTGGGGAGG CGTGAAGCGG TCCCGTGGAT AGAGATTCAT GCCTGTGTCC GCGCGTGTGT	600
GCGCGCGTAT AAATTGCCGA GAAGGGGAAA ACATCACAGG ACTTCTGCGA ATACCGGACT	660
GAAAATTGTA ATTCATCTGC CGCCGCCGCT GCCAAAAAAA AACTCGAGCT CTTGAGATCT	720
CCGGTTGGGA TTCCTGCGGA TTGACATTC TGTGAAGCAG AAGTCTGGGA ATCGATCTGG	780
AAATCCTCCT AATTTTTACT CCCTCTCCCC CCGACTCCTG ATTCATTGGG AAGTTTCAA	840
TCAGCTATAA CTGGAGAGTG CTGAAGATTG ATGGGATCGT TGCCTTATGC ATTTGTTTTG	900
GTTTTACAAA AAGGAACTT GACAGAGGAT CATGCTGTAC TTAAAAATA CAAGTAAGTC	960
TCGCACAGGA AATTGGTTTA ATGTAACTTT CAATGGAAAC CTTTGAGATT TTTACTTAA	1020
AGTGCATTCG AGTAAATTTA ATTTCCAGGC AGCTTAATAC ATTGTTTTTA GCCGTGTTAC	1080
TTGTAGTGTG TATGCCCTGC TTCACTCAG TGTGTACAGG GAAACGCACC TGATTTTTTA	1140
CTTATTAGTT TGTTTTTTCT TTAACCTTC AGCATCACAG AGGAAGTAGA CTGATATTAA	1200
CAATACTTAC TAATAATAAC GTGCCTCATG AATAAAGAT CCGAAAGGAA TTGGAATAAA	1260
AATTCCTGC GTCTCATGCC AAGAGGGAAA CACCAGAATC AAGTGTTCCG CGTGATTGAA	1320
GACACCCCT CGTCCAAGAA TGCAAAGCAC ATCCAATAAA ATAGCTGGAT TATAACTCCT	1380
CTTCTTTCTC TGGGGGCCGT GGGGTGGGAG CTGGGGCGAG AGGTGCCGTT GGCCCCCGTT	1440
GCTTTTCCTC TGGGAAGG ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC	1491
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn	
1 5 10	
CGG GAG ATA GTG ATG AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC	1539
Arg Glu Ile Val Met Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly	
15 20 25	

TAC GAG TGG GAT GCG GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC	1587
Tyr Glu Trp Asp Ala Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala	
30 35 40	
CCC GCA CCG GGC ATC TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA	1635
Pro Ala Pro Gly Ile Phe Ser Ser Gln Pro Gly His Thr Pro His Pro	
45 50 55	
GCC GCA TCC CGC GAC CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG	1683
Ala Ala Ser Arg Asp Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro	
60 65 70 75	
GCT GCC CCC GGC GCC GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT	1731
Ala Ala Pro Gly Ala Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro	
80 85 90	
GTG GTC CAC CTG GCC CTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC	1779
Val Val His Leu Ala Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg	
95 100 105	
TAC CGC GGC GAC TTC GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC	1827
Tyr Arg Gly Asp Phe Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro	
110 115 120	
TTC ACC GCG CGG GGA CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG	1875
Phe Thr Ala Arg Gly Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg	
125 130 135	
GAC GGG GTG AAC TGG GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGG	1923
Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly	
140 145 150 155	
GTC ATG TGT GTG GAG AGC GTC AAC CGG GAG ATG TCG CCC CTG GTG GAC	1971
Val Met Cys Val Glu Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp	
160 165 170	
AAC ATC GCC CTG TGG ATG ACT GAG TAC CTG AAC CGG CAC CTG CAC ACC	2019
Asn Ile Ala Leu Trp Met Thr Glu Tyr Leu Asn Arg His Leu His Thr	
175 180 185	
TGG ATC CAG GAT AAC GGA GGC TGG GAT GCC TTT GTG GAA CTG TAC GGC	2067
Trp Ile Gln Asp Asn Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly	
190 195 200	
CCC AGC ATG CCG CCT CTG TTT GAT TTC TCC TGG CTG TCT CTG AAG ACT	2115
Pro Ser Met Arg Pro Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr	
205 210 215	
CTG CTC AGT TTG GCC CTG GTG GGA GCT TGC ATC ACC CTG GGT GCC TAT	2163
Leu Leu Ser Leu Ala Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr	
220 225 230 235	
CTG AGC CAC AAG TGAAGTCAAC ATGCCTGCCC CAAACAAATA TGCAAAAGGT	2215
Leu Ser His Lys	
240	
TCACTAAAGC AGTAGAAATA ATATGCATTG TCAGTGATGT ACCATGAAAC AAAGCTGCAG	2275
GCTGTTTAAG AAAAAATAAC ACACATATAA ACATCACACA CACAGACAGA CACACACACA	2335
CACAACAATT AACAGTCTTC AGGCAAAACG TCGAATCAGC TATTTACTGC CAAAGGGAAA	2395



-63-

TATCATTTAT	TTTTTACATT	ATTAAGAAAA	AAGATTTATT	TATTTAAGAC	AGTCCCATCA	2455
AAACTCCGTC	TTTGGAATC	CGACCACTAA	TTGCCAAACA	CCGCTTCGTG	TGGCTCCACC	2515
TGGATGTTCT	GTGCCTGTAA	ACATAGATTC	GCTTTCCATG	TTGTTGGCCG	GATCACCATC	2575
TGAAGAGCAG	ACGGATGGAA	AAAGGACCTG	ATCATTGGGG	AAGCTGGCTT	TCTGGCTGCT	2635
GGAGGCTGGG	GAGAAGGTGT	TCATTCACTT	GCATTTCTTT	GCCCTGGGGG	CGTGATATTA	2695
ACAGAGGGAG	GGTTCCCGTG	GGGGGAAGTC	CATGCCTCCC	TGGCCTGAAG	AAGAGACTCT	2755
TTGCATATGA	CTCACATGAT	GCATACCTGG	TGGGAGGAAA	AGAGTTGGGA	ACTTCAGATG	2815
GACCTAGTAC	CCACTGAGAT	TTCCACGCCG	AAGGACAGCG	ATGGGAAAAA	TGCCCTTAAA	2875
TCATAGGAAA	GTATTTTTTT	AAGCTACCAA	TTGTGCCGAG	AAAAGCATTT	TAGCAATTTA	2935
TACAATATCA	TCCAGTACCT	TAAACCCTGA	TTGTGTATAT	TCATATATTT	TGGATACGCA	2995
CCCCCAACT	CCCAATACTG	GCTCTGTCTG	AGTAAGAAAC	AGAATCCTCT	GGAACCTGAG	3055
GAAGTGAACA	TTTCGGTGAC	TTCCGATCAG	GAAGGCTAGA	GTTACCCAGA	GCATCAGGCC	3115
GCCACAAGTG	CCTGCTTTTA	GGAGACCGAA	GTCCGCAGAA	CCTACCTGTG	TCCCAGCTTG	3175
GAGGCCTGGT	CCTGGAACCTG	AGCCGGGCCC	TCACTGGCCT	CCTCCAGGGA	TGATCAACAG	3235
GGTAGTGTGG	TCTCCGAATG	TCTGGAAGCT	GATGGATGGA	GCTCAGAATT	CCACTGTCAA	3295
GAAAGAGCAG	TAGAGGGGTG	TGGCTGGGCC	TGTCACCCTG	GGGCCCTCCA	GGTAGGCCCC	3355
TTTTCACGTG	GAGCATAGGA	GCCACGACCC	TTCTTAAGAC	ATGTATCACT	GTAGAGGGAA	3415
GGAACAGAGG	CCCTGGGCCT	TCCTATCAGA	AGGACATGGT	GAAGGCTGGG	AACGTGAGGA	3475
GAGGCAATGG	CCACGGCCCA	TTTTGGCTGT	AGCACATGGC	ACGTTGGCTG	TGTGGCCTTG	3535
GCCACCTGTG	AGTTTAAAGC	AAGGCTTTAA	ATGACTTTGG	AGAGGGTCAC	AAATCCTAAA	3595
AGAAGCATTG	AAGTGAGGTG	TCATGGATTA	ATTGACCCCT	GTCTATGGAA	TTACATGTAA	3655
AACATTATCT	TGTCACTGTA	GTTTGGTTTT	ATTGAAAAC	CTGACAAAAA	AAAAGTTCCA	3715
GGTGTGGAAT	ATGGGGGTTA	TCTGTACATC	CTGGGGCATT	AAAAAAAAAT	CAATGGTGGG	3775
GAACATAAAA	GAAGTAACAA	AAGAAGTGAC	ATCTTCAGCA	AATAAACTAG	GAAATTTTTT	3835
TTTCTTCCAG	TTTAGAATCA	GCCTTGAAAC	ATTGATGGAA	TAACCTGTG	GCATTATTGC	3895
ATTATATACC	ATTTATCTGT	ATTAACCTTG	GAATGTACTC	TGTTCAATGT	TTAATGCTGT	3955
GGTTGATATT	TCGAAAGCTG	CTTTAAAAAA	ATACATGCAT	CTCAGCGTTT	TTTTGTTTTT	4015
AATTGTATTT	AGTTATGGCC	TATACACTAT	TTGTGAGCAA	AGGTGATCGT	TTTCTGTTTG	4075
AGATTTTTAT	CTCTTGATTG	TTCAAAAGCA	TTCTGAGAAG	GTGAGATAAG	CCCTGAGTCT	4135
CAGCTACCTA	AGAAAAACCT	GGATGTCACT	GGCCACTGAG	GAGCTTTGTT	TCAACCAAGT	4195
CATGTGCATT	TCCACGTCAA	CAGAATTGTT	TATTGTGACA	GTTATATCTG	TTGTCCCTTT	4255

GACCTTGTTT CTTGAAGGTT TCCTCGTCCC TGGGCAATTC CGCATTTAAT TCATGGTATT	4315
CAGGATTACA TGCATGTTTG GTTAAACCCA TGAGATTCAT TCAGTTAAAA ATCCAGATGG	4375
CGAATGACCA GCAGATTCAA ATCTATGGTG GTTTGACCTT TAGAGAGTTG CTTTACGTGG	4435
CCTGTTTCAA CACAGACCCA CCCAGAGCCC TCCTGCCCTC CTTCCGCGGG GGCTTTCTCA	4495
TGGCTGTCCT TCAGGGTCTT CCTGAAATGC AGTGGTCGTT ACGCTCCACC AAGAAAGCAG	4555
GAAACCTGTG GTATGAAGCC AGACCTCCCC GCGGGGCCTC AGGGAACAGA ATGATCAGAC	4615
CTTTGAATGA TTCTAATTTT TAAGCAAAAT ATTATTTTAT GAAAGGTTTA CATTGTCAAA	4675
GTGATGAATA TGGAAATATCC AATCCTGTGC TGCTATCCTG CCAAATCAT TTTAATGGAG	4735
TCAGTTTGCA GTATGCTCCA CGTGGTAAGA TCCTCCAAGC TGCTTTAGAA GTAACAATGA	4795
AGAACGTGGA CGTTTTTAAT ATAAAGCCTG TTTTGTCTTT TGTGTGTGTT CAAACGGGAT	4855
TCACAGAGTA TTTGAAAAAT GTATATATAT TAAGAGGTCA CGGGGGCTAA TTGCTAGCTG	4915
GCTGCCTTTT GCTGTGGGGT TTTGTTACCT GGTTTTAATA ACAGTAAATG TGCCCAGCCT	4975
CTTGCCCCCA GAACTGTACA GTATTGTGGC TGCACTTGCT CTAAGAGTAG TTGATGTTGC	5035
ATTTTCCTTA TTGTTAAAA CATGTTAGAA GCAATGAATG TATATAAAAG C	5086

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 239 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Ala	His	Ala	Gly	Arg	Thr	Gly	Tyr	Asp	Asn	Arg	Glu	Ile	Val	Met
1				5					10					15	
Lys	Tyr	Ile	His	Tyr	Lys	Leu	Ser	Gln	Arg	Gly	Tyr	Glu	Trp	Asp	Ala
			20					25					30		
Gly	Asp	Val	Gly	Ala	Ala	Pro	Pro	Gly	Ala	Ala	Pro	Ala	Pro	Gly	Ile
		35					40					45			
Phe	Ser	Ser	Gln	Pro	Gly	His	Thr	Pro	His	Pro	Ala	Ala	Ser	Arg	Asp
	50					55					60				
Pro	Val	Ala	Arg	Thr	Ser	Pro	Leu	Gln	Thr	Pro	Ala	Ala	Pro	Gly	Ala
	65				70				75					80	
Ala	Ala	Gly	Pro	Ala	Leu	Ser	Pro	Val	Pro	Pro	Val	Val	His	Leu	Ala
				85					90					95	
Leu	Arg	Gln	Ala	Gly	Asp	Asp	Phe	Ser	Arg	Arg	Tyr	Arg	Gly	Asp	Phe
		100						105					110		

-65-

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly  
 115 120 125  
 Arg Phe Ala Thr Val Val Glu Leu Phe Arg Asp Gly Val Asn Trp  
 130 135 140  
 Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu  
 145 150 155 160  
 Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp  
 165 170 175  
 Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn  
 180 185 190  
 Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro  
 195 200 205  
 Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala  
 210 215 220  
 Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys  
 225 230 235

CLAIMS

1. Substantially pure nucleic acid which is a cell death-protective gene.
- 5 2. The nucleic acid of Claim 1, wherein the cell death-protective gene is the C. elegans ced-9 gene.
3. Substantially pure nucleic acid consisting essentially of all or a portion of the nucleotide sequence selected from the group consisting of:
  - 10 a) the nucleotide sequence shown in Figure 2 (SEQ ID NO:1); and
  - b) the nucleotide sequence shown in Figure 3 (SEQ ID NO:2).
4. Substantially pure nucleic acid encoding the amino acid sequence shown in Figure 4 (SEQ ID NO:3).
- 15 5. Isolated protein encoded by the C. elegans ced-9 gene.
6. The protein of Claim 5 consisting essentially of the amino acid sequence shown in Figure 4 (SEQ ID NO:3).
- 20 7. An antibody specifically reactive with the protein of Claim 5.
8. Substantially pure nucleic acid which is a mutated cell death-protective gene, wherein the mutation constitutively activates the gene.
- 25 9. The nucleic acid of Claim 8, wherein the cell death-protective gene is C. elegans ced-9.

-67-

10. Isolated protein encoded by the nucleic acid of Claim 9.
11. The nucleic acid of Claim 9, wherein the mutation is n1950.
- 5 12. The nucleic acid of Claim 11, wherein the mutation is associated with a glycine to glutamic acid change at codon 169.
13. The nucleic acid of Claim 8, wherein the cell death-protective gene is human bcl-2.
- 10 14. Isolated protein encoded by the nucleic acid of Claim 13.
15. The nucleic acid of Claim 13, wherein the mutation is associated with a glycine to glutamic acid change at codon 145.
- 15 16. Substantially pure nucleic acid which is a mutated cell death-protective gene, wherein the mutation inactivates the gene.
17. Isolated protein encoded by the nucleic acid of Claim 16.
- 20 18. The nucleic acid of Claim 16, wherein the cell death-protective gene is C. elegans ced-9.
19. The nucleic acid of Claim 18, wherein the mutation is selected from the group consisting of:
  - a) n2077;
  - 25 b) n2161; and
  - c) n1653ts.

20. The nucleic acid of Claim 18, wherein the mutation is associated with a change selected from the group consisting of:
- a) tyrosine to asparagine at codon 149; and
  - b) glutamine to termination at codon 160.
21. The nucleic acid of Claim 16, wherein the cell death-protective gene is human bcl-2.
22. Use of an agent which alters the activity of a cell death-protective gene (e.g. ced-9 or bcl-2) or a novel human cell death-protective gene structurally similar to ced-9 or bcl-2 in a cell (e.g. a nematode cell or a human cell), for the manufacture of a medicament for treating a condition in which the occurrence of cell death is at an abnormal rate.
23. Use according to Claim 22, wherein the cell death-protective gene is activated by a mutation, and protects against cell death.
24. Use according to Claim 23, wherein the cell death-protective gene is ced-9 and the mutation is n1950.
25. Use according to Claim 24, wherein the mutation is associated with a change from glycine to glutamic acid at codon 169.
26. Use according to Claim 23, wherein the cell death-protective gene is bcl-2, and the mutation is associated with a change from glycine to glutamic acid at codon 145.

-69-

27. Use according to Claim 22, wherein the cell death-protective gene is inactivated by a mutation, and does not provide protection against programmed cell death.
- 5 28. Use according to Claim 27, wherein the cell death-protective gene is C. elegans ced-9 and the mutation is selected from the group consisting of:
- 10 a) n2077;  
b) n2161; and  
c) n1653ts.
29. Use according to Claim 22, wherein the activity of the cell death-protective gene is increased, and the agent is selected from the group consisting of:
- 15 a) DNA comprising the cell death-protective gene;  
b) RNA encoded by the cell death-protective gene;  
c) protein encoded by the cell death-protective gene, or active portion thereof;  
d) an agent which mimics the activity of the cell death-protective gene;
- 20 e) an activator of the cell death protective gene; and  
f) an agonist of the cell death-protective gene.
30. Use according to Claim 22, wherein the activity of the cell death-protective gene is decreased, and the agent is selected from the group consisting of:
- 25 a) single stranded nucleic acid which is complementary to the mRNA of the cell death-protective gene;  
b) DNA which directs the expression of a);

- c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene;
  - 5 d) an inactivator of the cell death-protective gene; and
  - e) an antagonist of the cell death-protective gene.
31. Use according to Claim 22, wherein the medicament
- 10 is for treating in a population of cells a condition characterized by increased cell deaths, the agent is selected from the group consisting of:
- a) DNA comprising the cell death-protective gene;
  - b) RNA encoded by the cell death-protective gene;
  - 15 c) protein encoded by the cell death-protective gene, or active portion thereof;
  - d) an agent which mimics the activity of the cell death-protective gene;
  - e) an activator of the cell death protective
  - 20 gene; and
  - f) an agonist of the cell death-protective gene, under conditions appropriate for activity of the agent.
32. Use according to Claim 31, wherein the medicament
- 25 is for treating any of:
- a) myocardial infarction;
  - b) stroke;
  - c) traumatic brain injury;
  - d) neurodegenerative disease;
  - 30 e) muscular degenerative disease;
  - f) aging;
  - g) hypoxia;
  - h) ischemia;



-71-

- i) toxemia;
- j) infection, for example a virus infection e.g. human immunodeficiency virus infection; and
- k) hair loss.

5 33. Use of any of:

- a) single stranded nucleic acid which is complementary to the mRNA of the cell death-protective gene;
  - b) DNA which directs the expression of a);
  - 10 c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene;
  - d) an inactivator of the cell death-protective gene; and
  - 15 e) an antagonist of the cell death-protective gene,
- for the manufacture of a medicament for reducing a population of cells, comprising decreasing the activity of a cell death-protective gene (e.g. ced-9 or bcl-2 or a novel human cell death-protective gene structurally similar to ced-9 or bcl-2) in the treatment of for example any of:
- 20 a) neoplastic growth;
  - 25 b) cancerous tissue;
  - c) infected cells; and
  - d) autoreactive immune cells.

34. Use according to Claim 22, wherein the medicament is for treating a parasitic infection (e.g. a nematode infection) of a host animal, and the agent is adapted to decrease the activity of a cell death-protective gene specific to the parasite without harming the host animal.
- 30

35. Use according to Claim 34, wherein the agent is selected from:
- a) single stranded nucleic acid having all or a portion of the antisense sequence of the cell death-protective gene which is complementary to the mRNA of the gene; and
  - b) DNA which encodes a).
36. A method for altering cell death in a cell, comprising altering in the cell the activity of a cell death-protective gene.
37. The method of Claim 36, wherein the cell death-protective gene is ced-9 or bcl-2.
38. The method of Claim 36, wherein the cell is a nematode cell or a human cell.
39. The method of Claim 36, wherein cell death is prevented and the cell death-protective gene is activated and protects against cell death.
40. The method of Claim 36, wherein the cell death-protective gene is activated by a mutation.
41. The method of Claim 40, wherein the cell death-protective gene is ced-9 and the mutation is n1950.
42. The method of Claim 41, wherein the mutation is associated with a change from glycine to glutamic acid at codon 169.
43. The method of Claim 40, wherein the cell death-protective gene is bcl-2.

-73-

44. The method of Claim 43, wherein the mutation is associated with a change from glycine to glutamic acid at codon 145.
- 5 45. The method of Claim 36, wherein cell death is caused and the cell death-protective gene is inactivated and does not provide protection against programmed cell death.
46. The method of Claim 45, wherein the cell death-protective gene is inactivated by a mutation.
- 10 47. The method of Claim 46, wherein the cell death-protective gene is C. elegans ced-9 and the mutation is selected from the group consisting of:
- a) n2077;
- b) n2161; and
- 15 c) n1653ts.
48. The method of Claim 46, comprising exposing the cell to an agent which alters the activity of a cell death-protective gene in the cell under conditions appropriate for activity of the agent.
- 20 49. The method of Claim 48, wherein the cell death-protective gene is ced-9 or bcl-2.
50. The method of Claim 48, wherein the activity of the cell death-protective gene is increased, said method comprising exposing the cell to an agent
- 25 selected from the group consisting of:
- a) DNA comprising the cell death-protective gene;
- b) RNA encoded by the cell death-protective gene;
- c) protein encoded by the cell death-protective gene, or active portion thereof;

- d) an agent which mimics the activity of the cell death-protective gene;
- e) an activator of the cell death protective gene; and
- 5 f) an agonist of the cell death-protective gene.

51. The method of Claim 48, wherein the activity of the cell death-protective gene is decreased, said method comprising exposing the cell to an agent selected from the group consisting of:

- 10 a) single stranded nucleic acid which is complementary to the mRNA of the cell death-protective gene;
- b) DNA which directs the expression of a);
- 15 c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene;
- d) an inactivator of the cell death-protective gene; and
- 20 e) an antagonist of the cell death-protective gene.

52. A method for treating in a population of cells a condition characterized by increased cell deaths, comprising increasing in the population of cells  
25 the activity of a cell death-protective gene which functions in the cells.

53. The method of Claim 52, wherein the tissue is exposed to an agent selected from the group consisting of:

- 30 a) DNA comprising the cell death-protective gene;
- b) RNA encoded by the cell death-protective gene;

-75-

- c) protein encoded by the cell death-protective gene, or active portion thereof;
  - d) an agent which mimics the activity of the cell death-protective gene;
  - 5 e) an activator of the cell death protective gene; and
  - f) an agonist of the cell death-protective gene, under conditions appropriate for activity of the agent.
- 10 54. The method of Claim 52, wherein the cell death-protective gene is selected from the group consisting of:
- a) bcl-2;
  - 15 b) a novel human cell death-protective gene which is structurally similar to bcl-2; and
  - c) a novel human cell death-protective gene which is structurally similar to ced-9.
55. The method of Claim 53, wherein the condition is selected from the group consisting of:
- 20 a) myocardial infarction;
  - b) stroke;
  - c) traumatic brain injury;
  - d) neurodegenerative disease;
  - e) muscular degenerative disease;
  - 25 f) aging;
  - g) hypoxia;
  - h) ischemia;
  - i) toxemia;
  - j) infection; and
  - 30 k) hair loss.
56. The method of Claim 55, wherein the condition is a viral infection.

-76-

57. The method of Claim 56, wherein the viral infection is with human immunodeficiency virus.
58. A method for reducing a population of cells,  
5 comprising decreasing the activity of a cell death-protective gene which functions in the cells.
59. The method of Claim 58, wherein the cells are exposed to an agent selected from the group consisting of:
- 10 a) single stranded nucleic acid which is complementary to the mRNA of the cell death-protective gene;
- b) DNA which directs the expression of a);
- 15 c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene;
- d) an inactivator of the cell death-protective gene; and
- 20 e) an antagonist of the cell death-protective gene,  
under conditions appropriate for the activity of the agent.
60. The method of Claim 58, wherein the population of  
25 cells is selected from the group consisting of:
- a) neoplastic growth;
- b) cancerous tissue;
- c) infected cells; and
- d) autoreactive immune cells.
- 30 61. The method of Claim 58, wherein the cell death-protective gene is selected from the group consisting of:

-77-

- a) bcl-2;
  - b) a novel human cell death-protective gene which is structurally similar to bcl-2; and
  - c) a novel human cell death-protective gene which is structurally similar to ced-9.
- 5
62. A method for treating a parasitic infection of a host animal, comprising the steps of administering an agent which decreases the activity of a cell death-protective gene specific to the parasite and does not harm the host animal.
- 10
63. The method of Claim 62, wherein the agent is selected from:
- a) single stranded nucleic acid having all or a portion of the antisense sequence of the cell death-protective gene which is complementary to the mRNA of the gene; and
  - b) DNA which encodes a).
- 15
64. The method of Claim 62, wherein the parasite is a nematode.
- 20
65. A method of pest control, comprising decreasing the activity of a cell death-protective gene in the pest.
- 25
66. The method of Claim 65, comprising exposing the pest to an agent selected from the group consisting of:
- a) single stranded nucleic acid having all or a portion of the antisense sequence of the cell death-protective gene which is complementary to the mRNA of the gene;
  - b) DNA which directs the expression of a);
- 30

- c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene;
  - 5 d) an inactivator of the cell death-protective gene; and
  - e) an antagonist of the cell death-protective gene,
- 10 under conditions appropriate for the activity of the agent.
67. A method of biological containment of a recombinant organism, comprising introducing in the organism nucleic acid which is able to direct the expression of an agent which inactivates a cell death-
- 15 protective gene in the organism under predetermined conditions.
68. The method of Claim 67, wherein the agent is selected from the group consisting of:
- 20 a) single stranded nucleic acid which has all or a portion of the antisense sequence of the cell death-protective gene which is complementary to the mRNA of the gene;
  - b) DNA which directs the expression of a);
  - 25 c) a mutated cell death-protective gene which does not protect from cell death and which antagonizes the activity of the cell death-protective gene; and
  - d) an inactivator of the cell death-protective gene.
- 30 69. The method of Claim 68, wherein the agent kills the recombinant organism upon completion of a desired task by the organism.



-79-

70. A method for identifying a novel cell death-protective gene, comprising:
- a) obtaining a structurally similar gene by:
    - 1) identifying a gene which is structurally similar to a cell death-protective gene; or
    - 2) identifying a gene whose gene product is similar to the gene product of a cell death-protective gene; and
  - b) determining that the structurally similar gene protects cells in which it functions from cell death,
- thereby identifying a novel cell death-protective gene.
71. The method of Claim 70, wherein the cell death-protective gene is ced-9 or bcl-2.
72. Substantially pure nucleic acid which is a gene identified by the method of Claim 71.
73. The method of Claim 70, wherein step a) comprises the steps of:
- a) combining DNA with a nucleic acid probe comprising the cell death-protective gene, or a portion able to specifically hybridize to the cell death-protective gene, under conditions suitable for specific hybridization of the nucleic acid probe to complementary sequences; and
  - b) detecting specific hybridization of the nucleic acid probe to the DNA, wherein specific hybridization indicates that a structurally similar gene, or portion, is present in the DNA,

-80-

thereby identifying a gene which is structurally similar to a cell death-protective gene.

74. The method of Claim 73, wherein the DNA is a gene library.
- 5 75. The method of Claim 73, wherein the nucleic acid probe further comprises degenerate oligonucleotides derived from the amino acid sequence of the product of the cell death-protective gene.
- 10 76. The method of Claim 70, wherein step a) comprises the steps of:
- a) combining nucleic acid with primers comprising portions of the cell death-protective gene under conditions suitable for polymerase chain reaction; and
- 15 b) detecting specific DNA amplification, wherein specific DNA amplification produces a structurally similar gene, or portion, thereby identifying a gene which is structurally similar to a cell death-protective gene.
- 20 77. The method of Claim 76, wherein the primers further comprise degenerate oligonucleotides derived from the amino acid sequence of the product of the cell death-protective gene.
- 25 78. The method of Claim 70, wherein step a) comprises the steps of:

-81-

- 5           a)    combining an expression gene library with an antibody which binds specifically with the protein encoded by the cell death-protective gene under conditions suitable for specific antibody-antigen binding of the antibody to antigens expressed from the gene library; and
- 10           b)    detecting specific antibody-antigen binding, wherein specific antibody-antigen binding indicates that a structurally similar gene is present in the expression gene library, thereby identifying a gene which is structurally similar to a cell death-protective gene.
- 15           79.   The method of Claim 70, wherein step a) comprises searching a database of genes for a nucleotide sequence which is similar to the nucleotide sequence of the cell death-protective gene, thereby identifying a gene which is structurally similar to the cell death-protective gene.
- 20           80.   The method of Claim 70, wherein step a) comprises searching a database of proteins for an amino acid sequence which is similar to the amino acid sequence of the protein encoded by the cell death-protective gene, thereby identifying a gene which is structurally similar to the cell death-protective gene.
- 25           81.   The method of Claim 70, wherein step b) comprises the steps of:
- 30           a)    using the structurally similar gene and a nematode which lacks the activity of the ced-9 gene to produce a transgenic nematode; and

-82-

- 5           b)     determining in said transgenic nematode a decrease in cell deaths which occur during the development of the nontransgenic nematode, wherein a decrease in cell deaths indicates that the structurally similar gene protects cells in which it functions from cell death.
  
- 82.   The method of Claim 70, wherein step b) comprises the steps of:

  - 10           a)     introducing the structurally similar gene into cultured mammalian cells to produce transfected cells which express the gene; and
  - 15           b)     determining a decrease in cell deaths among the transfected cells under conditions which induce cell death, wherein a decrease in cell deaths indicates that the gene protects cells in which it functions from cell death.
  
- 83.   A bioassay for identifying a gene which has cell death-protective activity, comprising the steps of:

  - 20           a)     using DNA and a nematode which lacks the activity of the ced-9 gene to produce a transgenic nematode; and
  - 25           b)     determining in said transgenic nematode a decrease in cell deaths which occur during the development of the nontransgenic nematode, wherein a decrease in cell deaths indicates the activity of a cell death-protective gene in the DNA,

thereby identifying a gene which has cell death-protective activity.
  
- 30   84.   The bioassay of Claim 83, wherein the nematode underexpresses or expresses inactivated ced-9.

-83-

85. The bioassay of Claim 83, wherein the DNA is from an organism other than a nematode.
86. The bioassay of Claim 83, wherein the DNA is an expression gene library.
- 5 87. Substantially pure nucleic acid which is a cell death-protective gene identified by the bioassay of Claim 83.
88. Substantially pure nucleic acid which is a mutated cell death-protective gene identified by the  
10 bioassay of Claim 87.
89. A bioassay to identify a mutation in a cell death-protective gene which alters the activity of the gene, comprising the steps of:
- 15 a) using a cell death-protective gene having a mutation and a nematode which lacks ced-9 activity to produce a transgenic nematode; and
- b) comparing cell deaths which occur during the development of the transgenic nematode having the mutated gene with those which occur in a  
20 transgenic nematode having a non-mutated gene, wherein a difference in cell deaths indicates that the mutation alters the activity of the cell death-protective gene,
- thereby identifying a mutation in a cell death-protective gene which alters the activity of the  
25 gene.
90. Substantially pure nucleic acid which is a cell death-protective gene having a mutation identified by the bioassay of Claim 89.

91. A bioassay for identifying an agent which mimics the activity of a cell death-protective gene, comprising the steps of:
- 5 a) introducing an agent into a nematode which lacks the activity of ced-9; and
- b) detecting a decrease in cell deaths which occur in the nematode, wherein a decrease indicates that the agent mimics the activity of a cell death-protective gene,
- 10 thereby identifying an agent which mimics the activity of a cell death-protective gene.
92. The bioassay of Claim 91, wherein the nematode underexpresses or expresses inactivated ced-9.
93. The bioassay of Claim 91, wherein the agent is
- 15 introduced into the nematode by a method selected from: microinjection, diffusion, or ingestion.
94. An agent identified by the bioassay of Claim 91.
95. A bioassay for identifying an agent which affects the activity of a cell death-protective gene,
- 20 comprising the steps of:
- a) introducing an agent into a nematode which expresses a cell death-protective gene; and
- b) detecting a change in the pattern of cell
- 25 deaths which occur in the development of the nematode, wherein a change indicates that the agent affects the activity of the cell death-protective gene,
- thereby identifying an agent which affects the activity of a cell death-protective gene.

-85-

96. The bioassay of Claim 95, wherein the cell death-protective gene is ced-9.
97. The bioassay of Claim 95, wherein the cell death-protective gene is not ced-9 and the nematode is a transgenic nematode produced from said cell death-protective gene and a nematode which lacks ced-9 activity.
98. The bioassay of Claim 97, wherein the cell death-protective gene is bcl-2.
99. The bioassay of Claim 95, wherein the nematode overexpresses or underexpresses the cell death-protective gene.
100. The bioassay of Claim 95, wherein the nematode expresses an inactivated or constitutively activated form of the cell death-protective gene.
101. The bioassay of Claim 95, wherein the nematode underexpresses or expresses inactivated ced-9.
102. An agent identified by the method of Claim 95.
103. The agent of Claim 102, which is selected from the group consisting of:
- a) single stranded nucleic acid comprising all or a portion of the antisense sequence of a cell death-protective gene which is complementary to the mRNA encoded by the gene; and
  - b) DNA encoding a).
104. The agent of Claim 102, which is a gene which is not a cell death-protective gene.

105. The agent of Claim 102, which is a mutated cell death-protective gene which does not protect cells from cell death and which antagonizes the activity of cell death-protective genes which function.
- 5 106. A bioassay for identifying a cell death-protective gene, comprising the steps of:
- a) introducing DNA into cultured mammalian cells to produce transfected cells which express gene(s) in the DNA; and
  - 10 b) detecting cell deaths among the transfected cells under conditions which induce cell death, wherein a decrease in cell deaths indicates the activity of a cell death-protective gene in the DNA,
  - 15 thereby identifying a cell death-protective gene.
107. The bioassay of Claim 106, wherein the DNA is an expression gene library.
108. Substantially pure nucleic acid which is a gene identified by the method of Claim 106.
- 20 109. A bioassay for identifying a mutation in a cell death-protective gene which alters the activity of the gene, comprising the steps of:
- a) introducing a cell death-protective gene having a mutation into cultured mammalian cells to produce transfected cells which
  - 25 express the mutated gene; and



-87-

- 5           b)     comparing cell deaths which occur among the transfected cells under conditions which induce cell death to cell deaths which occur among cells transfected with the non-mutated gene, wherein a difference in cell deaths indicates that the mutation alters the activity of the gene, thereby identifying a mutation which alters the activity of a cell death-protective gene.
- 10   110. Substantially pure nucleic acid which is a cell death-protective gene having a mutation identified by the bioassay of Claim 109.
- 15   111. A bioassay for identifying an agent which mimics the activity of a cell death-protective gene, comprising the steps of:
- 20       a)     exposing cultured mammalian cells to an agent under conditions which induce cell death and which are appropriate for activity of the agent; and
- b)     detecting cell deaths in said exposed cells, wherein a decrease in cell deaths indicates that the agent protects cells from cell death, thereby identifying an agent which mimics the activity of a cell death-protective gene.
- 25   112. An agent identified by the bioassay of Claim 111.
113. A bioassay for identifying an agent which affects the activity of a cell death-protective gene, comprising the steps of:

- 5           a)    exposing cultured mammalian cells which are  
              protected by the activity of a cell death-  
              protective gene from conditions which induce  
              cell death to an agent under conditions which  
              are appropriate for activity of the agent; and  
              b)    detecting cell deaths in said exposed cells,  
                    wherein a change in cell deaths indicates that  
                    the agent affects the activity of the gene,  
10            thereby identifying an agent which affects the  
              activity of a cell death-protective gene.

114. An agent identified by the bioassay of Claim 113.

1/18

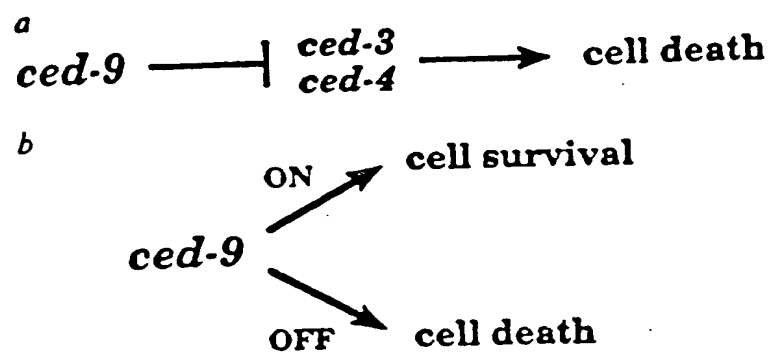


FIGURE 1



2/18

ATCGATAGTC GTCACCAAAT GGATTTTCCG ATTTCTCACT AGTCCATGGC TCACAATTTA	60
CAAAATCTCG AGAAAAGAAA GGATGCAAGG AGTATGAAGA GGTTCCGAAT CTAAATATTT	120
TAATTTAAAA AAATCAATTT CGAATTGAAA TTCAACTCCT ACTCGTTTTG AAAATGCCAA	180
TCCTTTAAGT AAACCTTCTGG ATCGCCCAT TCTTCCAGAA ATTCCTTCAA AGTAGTGGTT	240
TTGTACTGAT TTCCTCCGCA AAGAATAGGA ACTTTCGAAT CTCCTGGAGC GAAACGGGAT	300
TTTSATAACA AAAAATATC CAGACAAACC ATAGGACTTT TTCAAATATT CCTTATTTGG	360
CTGTCCATTT GGAAGCACCC AATCTTTAAC GCTGTCCAGC CAGAAGTGCT CCACTCGCCA	420
AGGATAAAAG GCTCATTTTT GAAGCCGAAT TTTACTAAAA TCTCTAGCCA TGGAGTCGAT	480
GGATCAGAAA TTCGAGGAAT TTTAGATTTC ATCTTGAAAT TTGCAATGGA AAAAATAATT	540
ATTCAAAGAA AATCACAGAA AATGCAACAA AAAAAACAA AAAAGAACAA AAAACAAGTC	600
GAAAAGTGC GCGGGGTCGT TTGCTGACGC ATCTCTTCAA ACGAGACGCG CTGCTGGCGA	660
CTTCTCGTGC CCTGTGCGTG CATTTCCGCA ACAAATTCA ACACTTGTTT TGAAACGCAC	720
CGCCCTGTTT CTTTTTCAA TTTTGATAAG AAAATCAGCA TTGTTTCAGG ATGATTAACA	780
TTCCAACTGC GATTCTGTGC CGCTTGGGCG CCAGATCGTC GATTTCCCGC TCCTTTGGAA	840
CATCGATCGT CACCAAGGTG GGGATTTTTT GAATTTTTCC GTGAAAATTG TTGATTTTTT	900
GTGTACGCAT GAAGGAGAAA TGTATAACAG ACACATTCTT TTCAATTAAT TATTTATAAT	960
ATTCACAGTC CGAGGCAAAG ACGCCAATCC AGAAGTTCCG ATGGGAATAC CTGTTGAAGC	1020
AGCGCTCCAA GAATCGCCCA ATCGCTCCAC ATCTCACCGT CTACCAGCCA CAATTGACCT	1080
GGATGCTCTC CGGATTCCAT AGAATCAGCG GTTGTGTAAT GGCCGGAACC CTTCTCGTCG	1140
GAGGAATCGG ATTCGCAGTT TGCCGTTTCG ATTTACCGC TTTTGTGGAT TTCATCCGTA	1200
GCTGGAACCT ACCATGCGCG GTGACCGCTG TCTTCAAGTA CATCATTGCT TTCCCCATCA	1260
TTTCCATAC TCTTAACGGA ATTCGCTTCT TAGGATTCTGA TTTGGCTAAG GGAGTCAATA	1320
ATGTTGGACA GGTAGGAGTT GAAATTATTA ATTTAATTGT TTTAAAATAA AAATTAATTT	1380
TCAGATCTAC AAATCGGGAT ATCTCGTATC TGGACTTTTCG GCTATTCTTG CTCTCGCCAT	1440
TGTCTTCAAC TCTTGCCAGA ACAAGAGCAA CAAGACTGCC TAGGCACAGA TGCTCCGCCT	1500
TCTTTTTTCT TACTCCGCCC CAGCCCTCGA CAATTCTCGT CAATTTACTT TTACCGTTGA	1560
TTTCTTCGAT TTTCTCTCTT TTCCGTAGAT TTACCTCTCC TCTTCGTTTT TTTTCTCTG	1620
TCTAGAATGT ATATTATGAT TATGAAAACG AATAAAATT TTAGATGACA CGCTGCACGG	1680
CGGACAACTC GCTGACGAAT CCGGCGTATC GCGACGAAC GATGGCGACT GCGGAGATGA	1740
AGGAGTTTCT GGGGATAAAA GGCACAGAGC CCACCGATTT TGAATCAAT AGTGATGCTC	1800
AGGACTTGCC ATCACCGAGT AGGCAGGCTT CGACGCGAAG AATGTCCATC GGAGAGTCAA	1860

FIGURE 2(a)



3/18

TTGATGGA	AAATCAATGAT	TGGGAAGAGC	CAAGGCTTGA	TATCGAGGGA	TTTGTGGTAA	1920
TTTTTTAATT	TTTTTTTGTA	AATAAAATTT	CCTGCTGCTT	CCAGGTCGAC	TATTTACACG	1980
ACCGAATCCG	GCAAAACGGA	ATGGAATGGT	TTGGAGCACC	GGGATTGCCG	TGTGGACTGC	2040
AACCGGAGCA	CGAAATGATG	CGAGTTATGG	GAACGATATT	CGAGAAGAAG	CACGCGGAAA	2100
ATTTTGAGAC	CTTCTGTGAG	CAGCTGCTCG	CAGTGCCCAG	AATCTCATTT	TCACTGTATC	2160
AGGATGTGGT	TCGGACGGTT	GGAAATGCAC	AGACAGATCA	ATGTCCAATG	TCTTATGGAC	2220
GTTTGGAAG	GGAGAAAATA	CTGAAAAAAA	GTTTGCAAAA	ATTGAAAAAT	TCGCCAGAAA	2280
GGTGGCAGAA	AAAACATTTG	CAAAAATTGT	TTGTTTTCTT	TCAGGAAATC	AGCAAAACTT	2340
GGTCAAAAAT	AGCCCAATTA	TGTGTCTTTT	TTGAAAGTTT	TCCATTAAAA	AACCACGAAT	2400
TTTGATCCCG	GATTGTAATT	TTTTTTGTTG	ATAAATTAGC	AGAAAACCTT	ACGAATTCGA	2460
TTAAAAACGT	TATTTTCTAT	TCGAATATTT	TTAAAGCATA	TTTTCTTGA	TTTGTATTTG	2520
CGAAAAAGAT	CTGCTGATTT	ATCAAAAATC	GGTTTTTAAA	TGTAAAATTT	GTGGAAAATA	2580
CATTAAAAAT	CGATTTTTGA	ACTTTTTTCT	TCGAAAAACA	GGTTTTTCTG	CTGATTTGCT	2640
GAACGAAAAA	CCCCAAAAAT	TCAATTTTCG	AACATTAAAA	ACCAGAAAAA	TCGTTTTTTT	2700
AAGCTTAATT	TTCCGCCAGA	AATGAACGAA	TTAAATTGCA	AATTTCTAAT	TTTCAGATAG	2760
GTCTAATCTC	GTTCCGGCGG	TTCGTAGCTG	CAAAAATGAT	GGAATCCGTG	GAAGTCAGG	2820
GACAAGTGCG	AAACCTCTTC	GTTTACACAT	CGCTGTTTAT	CAAAACGCGG	ATCCGCAACA	2880
ACTGGAAGGA	ACACAATCGG	AGCTGGGTAA	GGAGTATTTG	CATAGACATT	AGAAGTCAAT	2940
ATCCCCCTTT	CCCTAGTACC	CTTGACTTCC	CGGGGTGTTG	GTAAGCCGAT	AATTACAGGG	3000
TTCGGTAGCC	TCTTGGGGGG	ACAGCTGGAA	ACATATTCAA	GTATATTACT	GTTTATGATA	3060
ATGTTATTGT	TACGGGAATA	CAAAATTCGC	AGAATGCTAT	TTCAACAACAT	ATTTGACGCG	3120
CAAAATATCC	AGTAGAGAAA	ACTACAGTAA	TTCTTTAAAT	TTTAAAATTT	TTTACAATTA	3180
AAGAAAATAA	CCACTAATCA	AAAGAAATTA	ATTTCAAAAA	TCGAGCCCGT	AAATCGACTA	3240
CAGTAGGCAT	TTAAAGAATT	ACTGTAGTTT	TCGCTACGAG	ATATTTCCGC	CTCAAATATG	3300
TTGTGAAATA	CGCATTACAG	GATTTTTGTG	TTCCCCGGAA	TATGCTCTAA	AGCATTATTT	3360
GTGAAAATAA	AAAATCAAGA	AAAAAATTGC	AGGACGACTT	CATGACACTC	GGAAAACAAA	3420
TGAAAGAGGA	CTACGAACGA	GCAGAAGCTG	AAAAAGTGGG	ACGCCGGAAG	CAGAACAGAC	3480
GGTGGTCGAT	GATTGGCGCT	GGAGTAACAG	CTGGAGCCAT	TGGAATCGTT	GGAGTCGTCT	3540
TGTGTGGGCG	GATGATGTTT	AGCTTGAAGT	AACGTATTCA	ATTTGTGTAA	ATAATTAATT	3600
TATGTACAAC	TCCTTACATT	TGAATCTCAT	TTTTGCTCAC	TGATTCTCTC	ATCCTTTGAA	3660
CTGGAAGAAG	TGGGAAAGCT	AGGCCACAAA	TTACGGCTCT	CTGTGTCGAT	TTACGATTTT	3720

FIGURE 2(b)

SUBSTITUTE SHEET





4/18

ACTGCAATTT TTTCCGATTG CCTTTTTTTT TGGCCAAACC CTACTTCCGC GTAATATCAA	3780
CTTTTCCGTG TTCTGTACAT TTCGTCAAAA ACCCTGAAAC CCTAACTTTT CTCGCCGTGG	3840
CCTAGCCTCC CGCTTCTCTT CCACATTTCC AAAGTACCCC TGTATCTCAA TAATTCATCT	3900
TCACTTTAAC TGTCTCTTTT CGTGTGGCCT CTTCCTCACTC CCCCCAAATT CCTGTACGCG	3960
TACGCGACTT TGTATTTATT TTTTCAAAT TGTTTTCTCT CTACAACAAC AAAAAAACC	4020
GTTCTTTTAT TCAACCCTTT TTTCCGAACG AACTGCAAT TTTGATAATA GGCGTGCGCA	4080
AGAGAATCCG GTTTTCATTT TCGCCATCAC GTCATCCAAA AAAGTTTAGT AGGAAAATAT	4140
CATTTTTTAA TATAATGATT CATCTTTCTC GCCTCTTCTG TCTCGAGACG ACGGTCAATT	4200
CGATGGCCTT GAATTTTTTCG AAAACAAAAA TGTTTTTGTT TAGTGTAAC GATCCCCCG	4260
CCTTATCGCT GTTTCACCAT CAGATAGGCT CCGCCATTTG ATTCCCTTGA ATTTTGTCGG	4320
TATATAAAC AAAAAACGTT AGTGCACGAT TCAAAAAACA ACAATGCGTG CTTTACTATT	4380
CACCTCTGTT GTTCTTTTGG CTTTGGCTTT TGTTGAGGCA AAGAAGCAGA CTATCACTGT	4440
CAAGGGTACA ACTATTTGTA ATAAGAAGAG AATTCAGGMG RAGGTTACCT TTGGGAGAAA	4500
GATACTCGTG AGTTTTCAGT CTTGTTTAGC TTGAAACGGC TAAAAAGGA CTAAAAAGGC	4560
CTAAAAATTG AAGTTTTCCA CCTGTTTTCA AAAGAAAGCC GAATTGCACA GCTTTACACG	4620
AGATTTCTCA ATAATTTGTA TTTGAAATTT TCATATTCAT CCCCAAACGT TCTTTACACG	4680
AAATTTTGCG ATTTTGTAGC TTAATAACG ATACCTGGTC TCGACACGAA ACATTTTGT	4740
TAAATTCAAA AAGATGTGCG CCTTTAAAGA GTGCTGTAGT TTGAACTTC TGTGTGCG	4800
GACTTTTCAT CGATTTTTTCG TAGCGTTTTT TTATAAGAAA AATGTATTTA TTTATTCAAA	4860
AATTTAATTT TACCGAATCG CGAAAAACAA AATGAAGAAC ACCGATAAAA ATATCGCAGC	4920
AACAATAGTT TGAAATTACA GTACTCTTTT AAGGNGNCA CATTTCCTAT ATTTACACACA	4980
AACTTGTCGT GTCGNNCNG GGTATCGTCA TTTTGATGCA GAAATCAAGA AAATTGCATA	5040
TATGTTCAAA AAACCACAAT TATGGCGAAT TTCAAGCTTG AAACGAAAAT TCAGGAAATT	5100
CTAAAAATTA AAAAAAATC ATTCGAAATG TGAAATTTGA TATTCAACTT GAAGTCCATA	5160
TGGCAAATTT CGTCTATTCC GNNNTTCGAN NATTTTGTTT CACGTGGCCG CGAAAAGAGA	5220
AAGCACGANN ACTGATTTCT GGCAATTTTT TCCTGTACCG TGTCAATTAT TTGAACTCT	5280
AATAAGCTGG TATTTTCTG CTATTGACAA CTAAGTGAAT CCATAATTG CAATTATAAT	5340
ATTGACTTTT GATGTGTGGC TTAGAAAAA AAAACCAAAA ACCTCATCTA GCTTTAGGCT	5400
GCCAATATAT TCCTAGGACA TATAAAAAAC CCTTAAATTT CTCTGCAACA CCTACAAGCT	5460
ATCAAACGTA CTATTAGTAT TCAATTTTCC AGTCGACCCC GATGACAAGC TCGCCTCAAT	5520
GCAATCGAAC AAAGAAGGAG AGTTCTCACT TACCGGATCC GACGACGAGA TCACCTCAAT	5580

FIGURE 2(c)



5/18

TCTCTCCATA CCTCATAATC ACCCACAACCT GCAACGTGAA GAAGGCCCGA TGCAAGCGTG	5640
TTTCAGAGTA TTTGATTCCA AAGGAGAAGA TCGGTGGAAC CTATGATATG ACATACGTCA	5700
CTCTTGATAT TCTTTCCGCT AAAGACAAGG AGAAGTGCTA AGAAAATGTT TTTTTTGTTT	5760
GGTTTGCTTG TTTGGAAGGG AAGGACTTTC TATCTCTTTT AATTCAACAA TAAACTATTG	5820
GAAAACCGTT GAAATTTTAA CCTTGAACCTG TAAGAAAAGT TCGGTGATTA TGTTGACAAT	5880
TTTGCCAAGT ATATCTTTGT GGATATCACA ATAAACGAAG TCAAAGCACG AAATATTACG	5940
GAAACACAAA ATTAATGAGA ATGCGCAACA TATTTGACCG CAAAATATCT CGTAGCGAAA	6000
CTACAGTAAT TCTTCAAAAG ACTACTGTAG CGCTGTGTCG ATTTACGAGC TCGATTTTTG	6060
AAATGAATCA GACTAGAAGA AAAGGAGGAA AATATTGAAC ATCAATTGAA CATCAATTCA	6120
AAAAGTCGAA CCCTTGACTA CAGTAGTCTT CTAAAGAATT ACTGTAGTTT TCGCTACGAG	6180
ATATTTTGNG NGTCAAATAT GTTGNGCAAT ACGCATCCTC AGAATTGTGT GTTCTCGTAA	6240
TGTCTTGAAA ATTTTCCATT TCAACATCAA ATAAGCAAAT CTAAAAATGT GGGTTCTGCA	6300
GCGACCACTA TGA CTGTGAT CGTGGCAAGA CCCACTCAGA AACTACGTG TTCCTTTAAA	6360
CAAATACATT TTTAAGTATT GTAGGTATAA AAATTGTTGG CTAGCAGTCT AGGCTGCCTT	6420
TTTCAGTCGA CAAACTTCTA ATTTAATCGG CGGGTCTTCA AAAAGTCGTT TCTTTGAAAA	6480
TATAAAGCTT TATATATTTA TATATTAAAA ATTTTGATTA CATGATATCA AAAGCGACTA	6540
GTTTGTATAA AAATTATCAA	6560

FIGURE (2d)



6/18

TTT GAG ATG ACA CGC TGC ACG GCG GAC AAC TCG CTG ACG AAT CCG GCG Met Thr Arg Cys Thr Ala Asp Asn Ser Leu Thr Asn Pro Ala 1 5 10	48
TAT CGG CGA CGA ACG ATG GCG ACT GGC GAG ATG AAG GAG TTT CTG GGG Tyr Arg Arg Arg Thr Met Ala Thr Gly Glu Met Lys Glu Phe Leu Gly 15 20 25 30	96
ATA AAA GGC ACA GAG CCC ACC GAT TTT GGA ATC AAT AGT GAT GCT CAG Ile Lys Gly Thr Glu Pro Thr Asp Phe Gly Ile Asn Ser Asp Ala Gln 35 40 45	144
GAC TTG CCA TCA CCG AGT AGG CAG GCT TCG ACG CGA AGA ATG TCC ATC Asp Leu Pro Ser Pro Ser Arg Gln Ala Ser Thr Arg Arg Met Ser Ile 50 55 60	192
GGA GAG TCA ATT GAT GGA AAA ATC AAT GAT TGG GAA GAG CCA AGG CTT Gly Glu Ser Ile Asp Gly Lys Ile Asn Asp Trp Glu Glu Pro Arg Leu 65 70 75	240
GAT ATC GAG GGA TTT GTG GTC GAC TAT TTC ACG CAC CGA ATC CGG CAA Asp Ile Glu Gly Phe Val Val Asp Tyr Phe Thr His Arg Ile Arg Gln 80 85 90	288
AAC GGA ATG GAA TGG TTT GGA GCA CCG GGA TTG CCG TGT GGA GTG CAA Asn Gly Met Glu Trp Phe Gly Ala Pro Gly Leu Pro Cys Gly Val Gln 95 100 105 110	336
CCG GAG CAC GAA ATG ATG CGA GTT ATG GGA ACG ATA TTC GAG AAG AAG Pro Glu His Glu Met Met Arg Val Met Gly Thr Ile Phe Glu Lys Lys 115 120 125	384
CAC GCG GAA AAT TTT GAG ACC TTC TGT GAG CAG CTG CTC GCA GTG CCC His Ala Glu Asn Phe Glu Thr Phe Cys Glu Gln Leu Leu Ala Val Pro 130 135 140	432
AGA ATC TCA TTT TCA CTG TAT CAG GAT GTG GTT CGG ACG GTT GGA AAT Arg Ile Ser Phe Ser Leu Tyr Gln Asp Val Val Arg Thr Val Gly Asn 145 150 155	480
GCA CAG ACA GAT CAA TGT CCA ATG TCT TAT GGA CGT TTG ATA GGT CTA Ala Gln Thr Asp Gln Cys Pro Met Ser Tyr Gly Arg Leu Ile Gly Leu 160 165 170	528
ATC TCG TTC GGC GGT TTC GTA GCT GCA AAA ATG ATG GAA TCC GTG GAA Ile Ser Phe Gly Gly Phe Val Ala Ala Lys Met Met Glu Ser Val Glu 175 180 185 190	576
CTG CAG GGA CAA GTG CGA AAC CTC TTC GTT TAC ACA TCG CTG TTC ATC Leu Gln Gly Gln Val Arg Asn Leu Phe Val Tyr Thr Ser Leu Phe Ile 195 200 205	624
AAA ACG CGG ATC CGC AAC AAC TGG AAG GAA CAC AAT CGG AGC TGG GAC Lys Thr Arg Ile Arg Asn Asn Trp Lys Glu His Asn Arg Ser Trp Asp 210 215 220	672
GAC TTC ATG ACA CTC GGA AAA CAA ATG AAA GAG GAC TAC GAA CGA GCA Asp Phe Met Thr Leu Gly Lys Gln Met Lys Glu Asp Tyr Glu Arg Ala 225 230 235	720
GAA GCT GAA AAA GTG GGA CGC CGG AAG CAG AAC AGA CGG TGG TCG ATG Glu Ala Glu Lys Val Gly Arg Arg Lys Gln Asn Arg Arg Trp Ser Met 240 245 250	768

FIGURE 3(a)



ATT GGC GCT GGA GTA ACA GCT GGA GCC ATT GGA ATC GTT GGA GTC GTC	816
Ile Gly Ala Gly Val Thr Ala Gly Ala Ile Gly Ile Val Gly Val Val	
255 260 265 270	
GTG TGT GGG CGG ATG ATG TTC AGC TTG AAG TAACGTATTC AATTTGTGTA	866
Val Cys Gly Arg Met Met Phe Ser Leu Lys	
275 280	
ATAAATTAAT TTATGTACAA CTCCTTACAT TTGAATCTCA TTTTKGCTCA CTGATTCTCT	926
CATCCTTTTGA ACTGGAAGAA GTGGGAAAGC TAGGCCACAA ATTACGGCTC TCTGTGTCGA	986
TTTACGATTT TACTGCAATT TTTTCCGATT GCCTTTTTTT TTGGCCAAAC CCTACTTCCG	1046
CGTAATATCA ACTTTTCCGT GTTCTGTACA TTTCGTCAAA AACCCTGAAA CCCTAACTTT	1106
TCTCGCCGTG GCCTAGCCTC CCGCTTCTCT TCCACATTTT CAAAGTACCC CTGTATCTCA	1166
ATAATTTCATC TTCACTTTAA CTGTCTCTTT TCGTGTGGCC TCTTCCAACCT CCCCCCAAT	1226
TCCTGTACGC GTACGCGACT TTGTATTTAT TTTTTTCAAA TTGTTTTCTC TCTACAACAA	1286
CAAAAAAAAAAC GGTTCAAAAA AAAAAAAAAA	1315

FIGURE 3(b)





8/18

## B30 ORF 920610 Sequence

---

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
MTRCTADNSL	TNPAYRRRIM	ATGEMKEFLG	IKGTEPTDFG	INSDAQDLPS	50
PSRQASTRRM	SIGESIDGKI	NWEEPRLDI	EGFWVDYFTH	RIRQNGMEWF	100
GAPGLPCGVQ	PEHEMRVMG	TIFEKKHAEN	FETFCQQLA	VPRISFSLYQ	150
DWRTVGNAQ	TDQCPMSYGR	LIGLISFGGF	VAAKMESVE	LQGQVRNLFV	200
YTSLFIKTRI	RNNWKEHNRS	WDDFMTLGKQ	MKEDYERAEA	EKVGRRKQNR	250
RWSMIGAGVT	AGAIGIVGW	VGGRMFSLK			280

FIGURE 4



9/18

MTRCTADNSL TNPAYRRRTM ATGEMKEFLG IKGTEPTDFG INSDAQDLPS 50

PSRQASTRRM SIGESIDGKI NDWEEPRLDI EGFVVDYFTH RIRQNGMEWF 100  
n1653ts (TAT -> AAT) N

GAPGLPCGVQ PEHEMMRVMG TIFEKKHAEN FETFCEQLLA VPRISFSLYQ 150  
E (GGA -> GAA) n1950

DVVRTVGNAQ TDQCPMSYGR LIGLISFGGF VAAKMESVE LQGQVRNLFV 200  
↓  
STOP (CAG -> TAG) n2077

YTSLFIKTRI RNNWKEHNRS WDDFMTLGKQ MKEDYERAEA EKVGRRKQNR 250

RWSMIGAGVT AGAIGIVGVV VCGRMMFSLK 280

FIGURE 5



10/18

Gap Weight:	3.000	Average Match:	0.540
Length Weight:	0.100	Average Mismatch:	-0.396
Quality:	89.8	Length:	298
Ratio:	0.376	Gaps:	11
Percent Similarity:	47.059	Percent Identity:	23.077

```

1 ... MTRCTADN..... SLTNPAYRRRTMATGEMKEFLGIKGTEPT 37
  . . . . . | . . . . . | . . . . . | . . . . . | . . . . .
1 MAHAGRTGYDNREIVMKYIHYKLSORGYEW... DAGDVGAAPPGAAPAG 47

38 DFGINSDAODLPSPSRQASTRRMSIGESIDGKINDWEEPRLDIEGFVVDY 87
  | : . . . . . | : | | : . . . . . | : . . . . . |
48 IFSSQPGHTPHPAASRDPVARTSPLOTPAAPGAA..... 81

88 FTHRIRONGMEWFGAPGLPCGVOPHEMMRVMTIFEKKHAKNFETFCEQ 137
  : | : | . . . . . | : | . . . . . | : . . . . . |
82 ..... AGPALSPVPPVVHLALROAGDDFSRRYRGDFAEMSSQ 118

138 LLAVPRISFSLYQDVVRTVGNAOTDOCPMSYGR LIGLISFGGFVAAKME 187
  | : . . . . . | : . . . . . | : . . . . . | : . . . . . |
119 LHLTPFTARGRFATVVEELFRD.... GVNWGRIVAFFEFGGVMC... VE 160
    conserved residue mutated in n1950 →
188 SV.. ELOGQVRNLFVYTSLFIKTRIRNNWKEHNRSWDDFMTL. GKOMKE. 233
  | | | : . . . . . | : . . . . . | : . . . . . | : . . . . . |
161 SVNREMSPLVDNIALWMTEYLNRL. HTWIQDNGGWDAFVELYGPSMRPL 209

234 . DYERAEAEKVGRRKONRRWSMIGAGVTAGAIGIVGVVVCGRMMFSLK 280
  | : . . . . . | : . . . . . | : . . . . . | : . . . . . |
210 FDFSWLSLKTLL..... LSLALVGACITLGAY..... LSHK 239

```

FIGURE 6



11/18

1 GCGCCCGCCC CTCCGCGCCG CCTGCCCCGC CGCCCGCCGC GCTCCCGCCC  
51 GCCGCTCTCC GTGGCCCCGC CGCGCTGCCG CCGCCGCCGC TGCCAGCGAA  
101 GGTGCCGGGG CTCCGGGCCC TCCCTGCCGG CGGCCGTCAG CGCTCGGAGC  
151 GAACTGCGCG ACGGGAGGTC CGGGAGGCGA CCGTAGTCGC GCCGCCGCGC  
201 AGGACCAGGA GGAGGAGAAA GGGTGCGCAG CCCGGAGGCG GGGTGCGCCG  
251 GTGGGGTGCA GCGGAAGAGG GGGTCCAGGG GGGAGAACTT CGTAGCAGTC  
301 ATCCTTTTTTA GGAAAAGAGG GAAAAAATAA AACCCTCCCC CACCACCTCC  
351 TTCTCCCCAC CCCTCGCCGC ACCACACACA GCGCGGGCTT CTAGCGCTCG  
401 GCACCGGCGG GCCAGGCGCG TCCTGCCTTC ATTTATCCAG CAGCTTTTCG  
451 GAAAATGCAT TTGCTGTTCG GAGTTTAATC AGAAGACGAT TCCTGCCTCC

FIGURE 7 (a)





12/18

501 GTCCCCGGCT CCTTCATCGT CCCATCTCCC CTGTCTCTCT CCTGGGGAGG  
551 CGTGAAGCGG TCCCGTGGAT AGAGATTCAT GCCTGTGTCC GCGCGTGTGT  
601 GCGCGCGTAT AAATTGCCGA GAAGGGGAAA ACATCACAGG ACTTCTGCGA  
651 ATACCGGACT GAAAATTGTA ATTCATCTGC CGCCGCCGCT GCCAAAAAAA  
701 AACTCGAGCT CTTGAGATCT CCGGTTGGGA TTCCTGCGGA TTGACATTTT  
751 TGTGAAGCAG AAGTCTGGGA ATCGATCTGG AAATCCTCCT AATTTTTACT  
801 CCCTCTCCCC CCGACTCCTG ATTCATTGGG AAGTTTCAAA TCAGCTATAA  
851 CTGGAGAGTG CTGAAGATTG ATGGGATCGT TGCCTTATGC ATTTGTTTTG  
901 GTTTTACAAA AAGGAACTT GACAGAGGAT CATGCTGTAC TTA AAAAATA  
951 CAAGTAAGTC TCGCACAGGA AATTGGTTTA ATGTAACTTT CAATGGAAAC  
1001 CTTTGAGATT TTTTACTTAA AGTGCATTCT AGTAAATTTA ATTTCCAGGC  
1051 AGCTTAATAC ATTGTTTTTA GCCGTGTTAC TTGTAGTGTG TATGCCCTGC  
1101 TTCACTCAG TGTGTACAGG GAAACGCACC TGATTTTTTA CTTATTAGTT  
1151 TGTTTTTTCT TTAACCTTTC AGCATCACAG AGGAAGTAGA CTGATATTAA  
1201 CAATACTTAC TAATAATAAC GTGCCTCATG AAATAAAGAT CCGAAAGGAA  
1251 TTGGAATAAA AATTTCTGTC GTCTCATGCC AAGAGGGAAA CACCAGAATC  
1301 AAGTGTTCCT CGTGATTGAA GACACCCCTT CGTCCAAGAA TGCAAAGCAC  
1351 ATCCAATAAA ATAGCTGGAT TATAACTCCT CTTCTTTCTC TGGGGGCCGT  
1401 GGGGTGGGAG CTGGGGCGAG AGGTGCCGTT GGCCCCCGTT GCTTTTCCTC  
1451 TGGGAAGGAT GGCGCACGCT GGGAGAACGG GGTACGACAA CCGGGAGATA  
1501 GTGATGAAGT ACATCCATTA TAAGCTGTCT CAGAGGGGCT ACGAGTGGGA  
1551 TCGGGGAGAT GTGGGCGCCG CGCCCCCGGG GGCCGCCCCC GCACCGGGCA  
1601 TCTTCTCCTC CCAGCCCGGG CACACGCCCC ATCCAGCCGC ATCCCGCGAC  
1651 CCGGTCGCCA GGACCTCGCC GCTGCAGACC CCGGCTGCCC CCGGCGCCGC  
1701 CGCGGGGCCT GCGCTCAGCC CCGTGCCACC TGTGGTCCAC CTGGCCCTCC  
1751 GCCAAGCCCG CGACGACTTC TCCCGCCGCT ACCGCGGCGA CTTGCGCGAG  
1801 ATGTCCAGCC AGCTGCACCT GACGCCCTTC ACCGCGCGGG GACGCTTTGC  
1851 CACGGTGGTG GAGGAGCTCT TCAGGGACGG GGTGAACTGG GGGAGGATTG  
1901 TGGCCTTCTT TGAGTTCGGT GGGGTCATGT GTGTGGAGAG CGTCAACCGG

FIGURE 7 (b)



13/18

1951 GAGATGTCGC CCCTGGTGGG CAACATCGCC CTGTGGATGA CTGAGTACCT  
2001 GAACCGGCAC CTGCACACCT GGATCCAGGA TAACGGAGGC TGGGATGCCT  
2051 TTGTGGAAC GTACGGCCCC AGCATGCGGC CTCTGTTTGA TTTCTCCTGG  
2101 CTGTCTCTGA AGACTCTGCT CAGTTTGGCC CTGGTGGGAG CTTGCATCAC  
2151 CCTGGGTGCC TATCTGAGCC ACAAGTGAAG TCAACATGCC TGGCCCAAAC  
2201 AAATATGCAA AAGGTTCACT AAAGCAGTAG AAATAATATG CATTGTCAGT  
2251 GATGTACCAT GAAACAAAGC TGCAGGCTGT TTAAGAAAAA ATAACACACA  
2301 TATAAACATC ACACACACAG ACAGACACAC ACACACACAA CAATTAACAG  
2351 TCTTCAGGCA AAACGTCGAA TCAGCTATTT ACTGCCAAAG GGAAATATCA  
2401 TTTATTTTTT ACATTATTAA GAAAAAAGAT TTATTTATTT AAGACAGTCC  
2451 CATCAAAACT CCGTCTTTGG AAATCCGACC ACTAATTGCC AACACCCGCT  
2501 TCGTGTGGCT CCACCTGGAT GTTCTGTGCC TGTAAACATA GATTGCTTT  
2551 CCATGTTGTT GGCCGGATCA CCATCTGAAG AGCAGACGGA TGGAAAAAGG  
2601 ACCTGATCAT TGGGGAAGCT GGCTTTCCTGG CTGCTGGAGG CTGGGGAGAA  
2651 GGTGTTCACT CACTTGCATT TCTTTGCCCT GGGGGCGTGA TATTAACAGA  
2701 GGGAGGGTTC CCGTGGGGGG AAGTCCATGC CTCCCTGGCC TGAAGAAGAG  
2751 ACTCTTTGCA TATGACTCAC ATGATGCATA CCTGGTGGGA GGAAAAGAGT  
2801 TGGGAACTTC AGATGGACCT AGTACCCACT GAGATTTCCA CGCCGAAGGA  
2851 CAGCGATGGG AAAAATGCCC TTAAATCATA GGAAAGTATT TTTTAAAGCT  
2901 ACCAATTGTG CCGAGAAAAG CATTTTAGCA ATTTATACAA TATCATCCAG  
2951 TACCTTAAAC CCTGATTGTG TATATTCATA TATTTTGGAT ACGCACCCCC  
3001 CAACTCCCAA TACTGGCTCT GTCTGAGTAA GAAACAGAAT CCTCTGGAAC  
3051 TTGAGGAAGT GAACATTTCC GTGACTTCCG ATCAGGAAGG CTAGAGTTAC  
3101 CCAGAGCATC AGGCCGCCAC AAGTGCCTGC TTTTAGGAGA CCGAAGTCCG  
3151 CAGAACCTAC CTGTGTCCCA GCTTGGAGGC CTGGTCTTGG AACTGAGCCG  
3201 GGCCCTCACT GGCCCTCTCC AGGGATGATC AACAGGGTAG TGTGGTCTCC  
3251 GAATGTCTGG AAGCTGATGG ATGGAGCTCA GAATTCCACT GTCAAGAAAG  
3301 AGCAGTAGAG GGGTGTGGCT GGGCCTGTCA CCCTGGGGCC CTCCAGGTAG  
3351 GCCCCTTTTC ACGTGGAGCA TAGGAGCCAC GACCCTTCTT AAGACATGTA

FIGURE 7 (c)



14/18

```

3401  TCACTGTAGA GGAAGGAAC AGAGGCCCTG GGCCTTCCTA TCAGAAGGAC
3451  ATGGTGAAGG CTGGGAACGT GAGGAGAGGC AATGGCCACG GCCCATTTTG
3501  GCTGTAGCAC ATGGCACGTT GGCTGTGTGG CCTTGGCCAC CTGTGAGTTT
3551  AAAGCAAGGC TTTAAATGAC TTTGGAGAGG GTCACAAATC CTAAAAGAAG
3601  CATTGAAGTG AGGTGTCATG GATTAATTGA CCCCTGTCTA TGAATTACA
3651  TGTA AACAT TATCTTGTC CTGTAGTTTG GTTTTATTTG AAAACCTGAC
3701  AAAAAAAAAA TTCCAGGTGT GGAATATGGG GGTATCTGT ACATCCTGGG
3751  GCATTAAAAA AAAATCAATG GTGGGGAAC ATAAAGAAGT AACAAAAGAA
3801  GTGACATCTT CAGCAAATAA ACTAGGAAAT TTTTTTTTCT TCCAGTTTAG
3851  AATCAGCCTT GAAACATTGA TGAATAACT CTGTGGCATT ATTGCATTAT
3901  ATACCATTTA TCTGTATTAA CTTTGGAATG TACTCTGTTT AATGTTTAAT
3951  GCTGTGGTTG ATATTTCGAA AGCTGCTTTA AAAAAATACA TGCATCTCAG
4001  CGTTTTTTTG TTTTAAATG TATTTAGTTA TGGCCTATAC ACTATTTGTG
4051  AGCAAAGGTG ATCGTTTTCT GTTTGAGATT TTTATCTCTT GATTCTTCAA
4101  AAGCATCTG AGAAGGTGAG ATAAGCCCTG AGTCTCAGCT ACCTAAGAAA
4151  AACCTGGATG TCACTGGCCA CTGAGGAGCT TTGTTTCAAC CAAGTCATGT
4201  GCATTTCCAC GTCAACAGAA TTGTTTATTG TGACAGTTAT ATCTGTTGTC
4251  CCTTTGACCT TGTTCCTTGA AGGTTTCCTC GTCCCTGGGC AATCCGCAT
4301  TTAATTCATG GTATTCAGGA TTACATGCAT GTTTGGTTAA ACCCATGAGA
4351  TTCAATCAGT TAAAAATCCA GATGGCGAAT GACCAGCAGA TTCAAATCTA
4401  TGGTGGTTTG ACCTTTAGAG AGTTGCTTTA CGTGGCCTGT TTCAACACAG
4451  ACCCACCAG AGCCCTCCTG CCCTCCTTCC GCGGGGGCTT TCTCATGGCT
4501  GTCCTTCAGG GTCTTCCTGA AATGCAGTGG TCGTTACGCT CCACCAAGAA
4551  AGCAGGAAAC CTGTGGTATG AAGCCAGACC TCCCCGGCGG GCCTCAGGGA
4601  ACAGAATGAT CAGACCTTTG AATGATTCTA ATTTTAAAGC AAAATATTAT
4651  TTTATGAAAG GTTTACATTG TCAAAGTGAT GAATATGGAA TATCCAATCC
4701  TGTGCTGCTA TCCTGCCAAA ATCATTTTAA TGGAGTCAGT TTGCAGTATG
4751  CTCCACGTGG TAAGATCCTC CAAGCTGCTT TAGAAGTAAC AATGAAGAAC
4801  GTGGACGTTT TTAATATAAA GCCTGTTTTG TCTTTTGTG TTGTTCAAAC

```

FIGURE 7 (d)



15/18

4851 GGGATTCACA GAGTATTTGA AAAATGTATA TATATTAAGA GGTACAGGGG  
4901 GCTAATTGCT AGCTGGCTGC CTTTGTCTGT GGGGTTTTGT TACCTGGTTT  
4951 TAATAACAGT AAATGTGCCC AGCCTCTTGG CCCCAGAACT GTACAGTATT  
5001 GTGGCTGCAC TTGCTCTAAG AGTAGTTGAT GTTGCATTTT CCTTATTGTT  
5051 AAAAACATGT TAGAAGCAAT GAATGTATAT AAAAGC

FIGURE 7 (e)





16/18

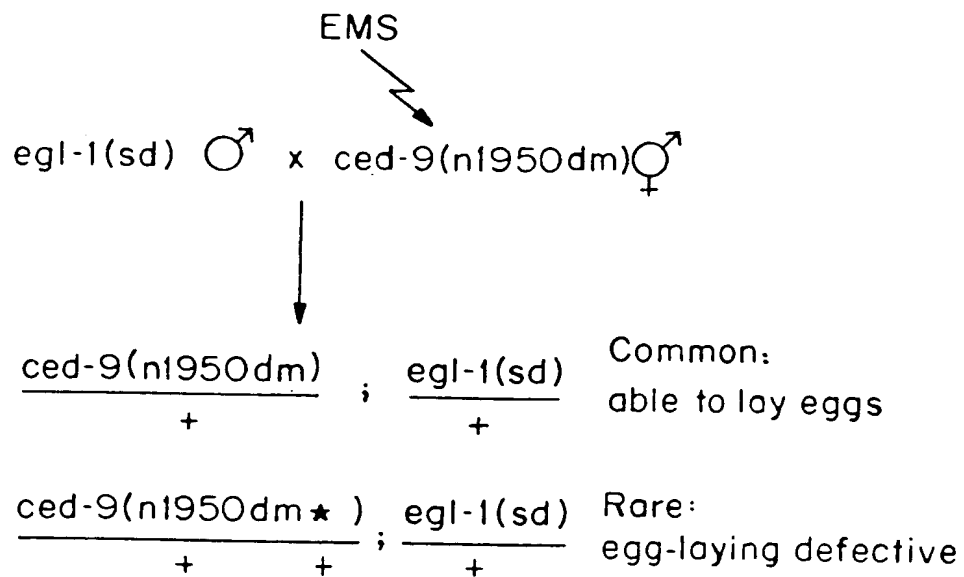


FIG. 8



17/18

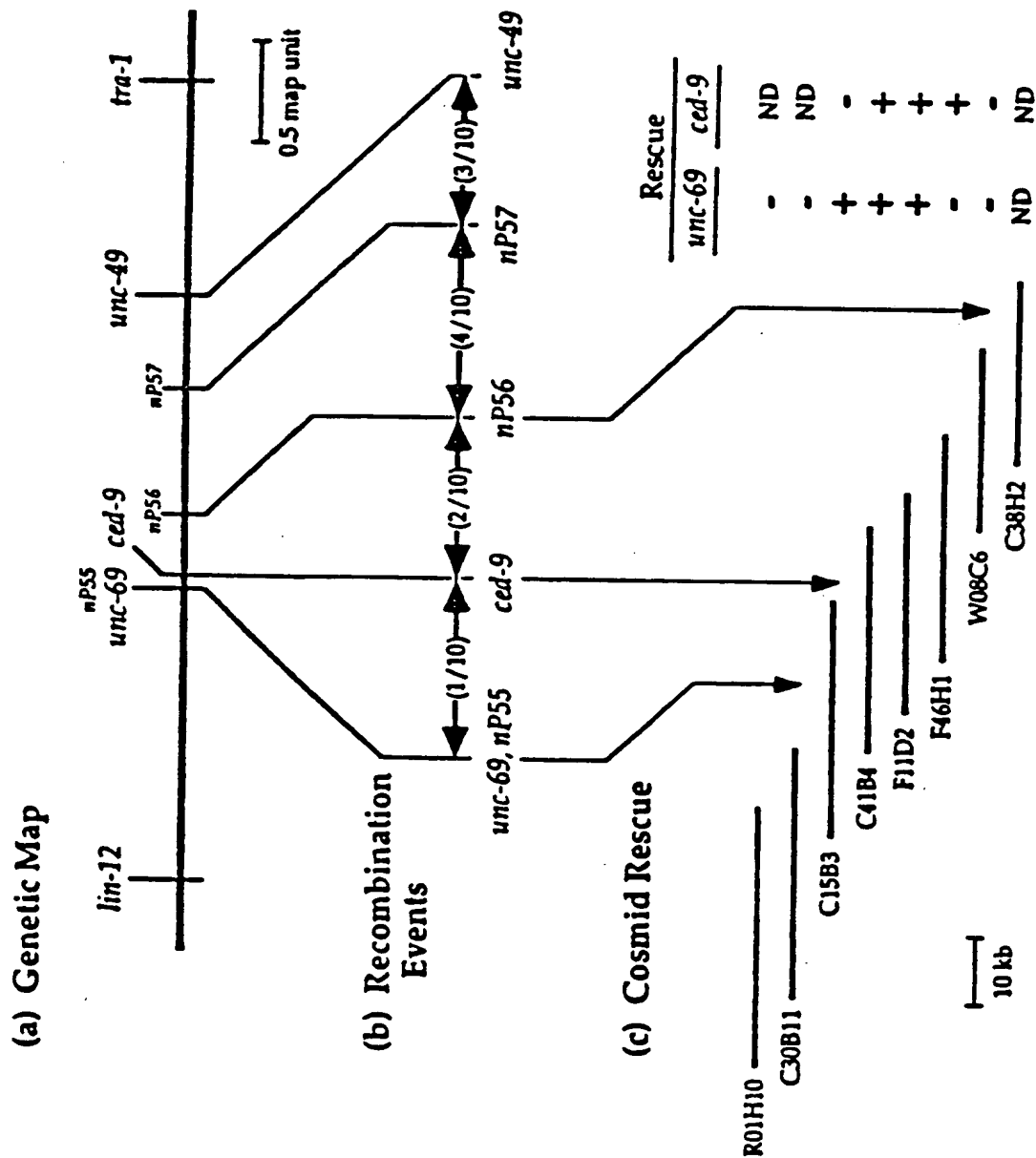
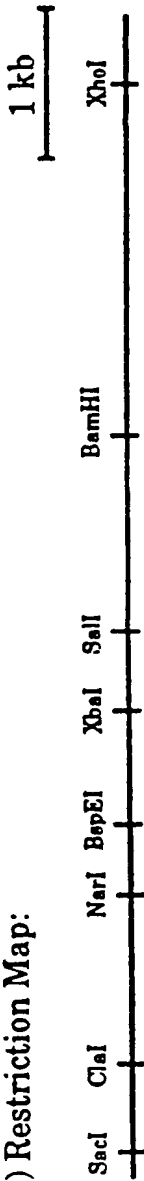


FIGURE 9



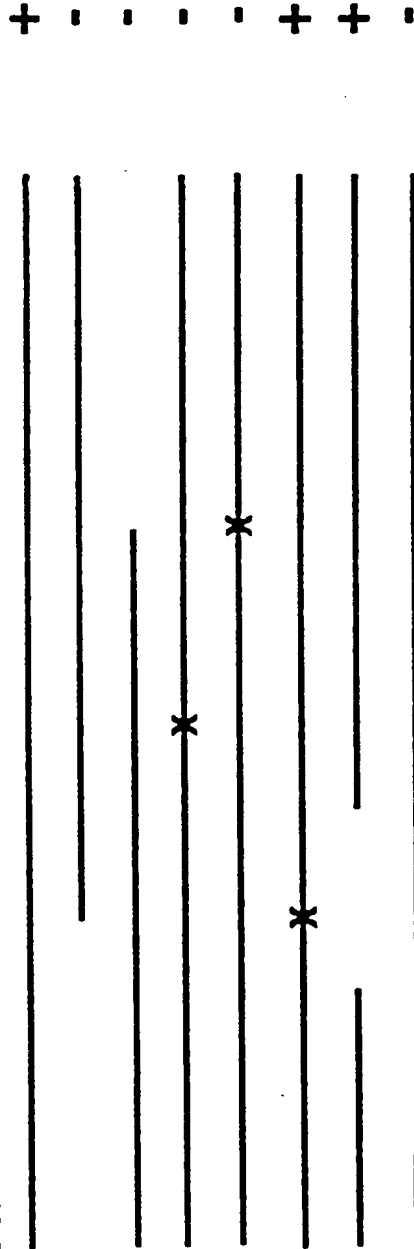
18/18

(a) Restriction Map:



Rescue

(b) Rescue:



(c) Transcripts (cDNAs):

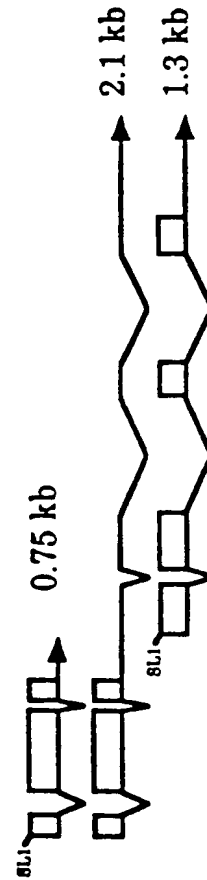


FIGURE 10



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/05651

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; A61K31/70;	C12N15/11; A61K37/02;	C12Q1/68; G01N33/577	C07K13/00
------------------------------------	--------------------------	-------------------------	-----------

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C12Q ; A61K G01N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	NATURE vol. 356, 9 April 1992, MACMILLAN JOURNALS LTD., LONDON, UK; pages 494 - 499 M.O. HENGARTNER ET AL. 'Caenorhabditis elegans gene ced-9 protects cells from programmed cell death' see page 494, left column, line 1 - page 499, right column, line 46 ---	1-12, 18-20, 22-25, 27-42, 45-114
Y	NATURE vol. 335, 8 September 1988, MACMILLAN JOURNALS LTD., LONDON, UK; pages 184 - 186 A. COULSON ET AL. 'Genome linking with yeast artificial chromosomes' see page 184, left column, line 1 - page 186, left column, line 22 ---	1-12, 18-20, 22-25, 27-42, 45-114
	-/-	

<sup>10</sup> Special categories of cited documents : <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search  04 OCTOBER 1993	Date of Mailing of this International Search Report  14 -10- 1993
International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer  HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>NATURE vol. 335, 28 September 1988, MACMILLAN JOURNALS LTD., LONDON, UK; pages 440 - 442 D.L. VAUX ET AL. 'Bcl-2 gene promotes haemopoietic cell survival and coöperates with c-myc to immortalize pre-B cells' cited in the application see page 440, left column, line 1 - page 442, right column, line 15 ---</p>	13-17, 21-23, 26-40, 43-46, 48-114
Y	<p>CELL vol. 67, no. 5, 29 November 1991, CELL PRESS, CAMBRIDGE, MA, US; pages 889 - 899 A. STRASSER ET AL. 'Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship' cited in the application see page 890, left column, paragraph 2 - page 895, left column, paragraph 1 ---</p>	13-17, 21-23, 26-40, 43-46, 48-114
Y	<p>CELL vol. 67, no. 5, 29 November 1991, CELL PRESS, CAMBRIDGE, MA, US; pages 879 - 888 C.S. SENTMAN ET AL. 'Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes' cited in the application see page 879, left column, line 1 - page 880, left column, line 10 ---</p>	13-17, 21-23, 26-40, 43-46, 48-114
Y	<p>ANNUAL REVIEW OF CELL BIOLOGY vol. 7, 1991, ANNUAL REVIEW INC., CALIFORNIA, US; pages 663 - 698 R.E. ELLIS ET AL. 'Mechanisms and functions of cell death' cited in the application the whole document ---</p>	1-114
A	<p>EP, A, 0 252 685 (THE WISTAR CORPORATION) 13 January 1988 the whole document ---</p>	-
P, Y	<p>WO, A, 9 300 909 (REGENERON PHARMACEUTICALS, INC.) 21 January 1993 see claims 1-98 see page 31, line 15 - page 41, line 27 ---</p>	52-61

-/--



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	SCIENCE vol. 258, 18 December 1992, AAAS, WASHINGTON, DC, US; pages 1955 - 1957 D.L. VAUX ET AL. 'Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2' see page 1955, left column, line 1 - page 1956, middle column, line 28 -----	13-17, 21-23, 26-40, 43-46, 48-114

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/05651

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 36-61 and 65-69 (as for as they concern in vivo treatment of human or animals) and claims 62-64 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9305651  
SA 76373

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

04/10/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0252685	13-01-88	US-A- 5015568	14-05-91
		AU-B- 602704	25-10-90
		AU-A- 7532887	18-02-88
		DE-A- 3786200	22-07-93
		JP-A- 63100379	02-05-88
		US-A- 5202429	13-04-93
-----			
WO-A-9300909	21-01-93	AU-A- 2322392	11-02-93
-----			

